

# O<sub>2</sub> deprivation inhibits Ca<sup>2+</sup>-activated K<sup>+</sup> channels via cytosolic factors in mice neocortical neurons

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Received for publication May 10, 1999, and accepted in revised form July 27, 1999.

O<sub>2</sub> deprivation induces membrane depolarization in mammalian central neurons. It is possible that this anoxia-induced depolarization is partly mediated by an inhibition of K<sup>+</sup> channels. We therefore performed experiments using patch-clamp techniques and dissociated neurons from mice neocortex. Three types of K<sup>+</sup> channels were observed in both cell-attached and inside-out configurations, but only one of them was sensitive to lack of O<sub>2</sub>. This O<sub>2</sub>-sensitive K<sup>+</sup> channel was identified as a large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel (BK<sub>Ca</sub>), as it exhibited a large conductance of 210 pS under symmetrical K<sup>+</sup> (140 mM) conditions, a strong voltage-dependence of activation, and a marked sensitivity to Ca<sup>2+</sup>. A low-O<sub>2</sub> medium (PO<sub>2</sub> = 10–20 mmHg) markedly inhibited this BK<sub>Ca</sub> channel open probability in a voltage-dependent manner in cell-attached patches, but not in inside-out patches, indicating that the effect of O<sub>2</sub> deprivation on BK<sub>Ca</sub> channels of mice neocortical neurons was mediated via cytosol-dependent processes. Lowering intracellular pH (pH<sub>i</sub>), or cytosolic addition of the catalytic subunit of a cAMP-dependent protein kinase A in the presence of Mg-ATP, caused a decrease in BK<sub>Ca</sub> channel activity by reducing the sensitivity of this channel to Ca<sup>2+</sup>. In contrast, the reducing agents glutathione and DTT increased single BK<sub>Ca</sub> channel open probability without affecting unitary conductance. We suggest that in neocortical neurons, (a) BK<sub>Ca</sub> is modulated by O<sub>2</sub> deprivation via cytosolic factors and cytosol-dependent processes, and (b) the reduction in channel activity during hypoxia is likely due to reduced Ca<sup>2+</sup> sensitivity resulting from cytosolic alterations such as in pH<sub>i</sub> and phosphorylation. Because of their large conductance and prevalence in the neocortex, BK<sub>Ca</sub> channels may be considered as a target for pharmacological intervention in conditions of acute anoxia or ischemia.

*J. Clin. Invest.* **104**:577–588 (1999).

## Introduction

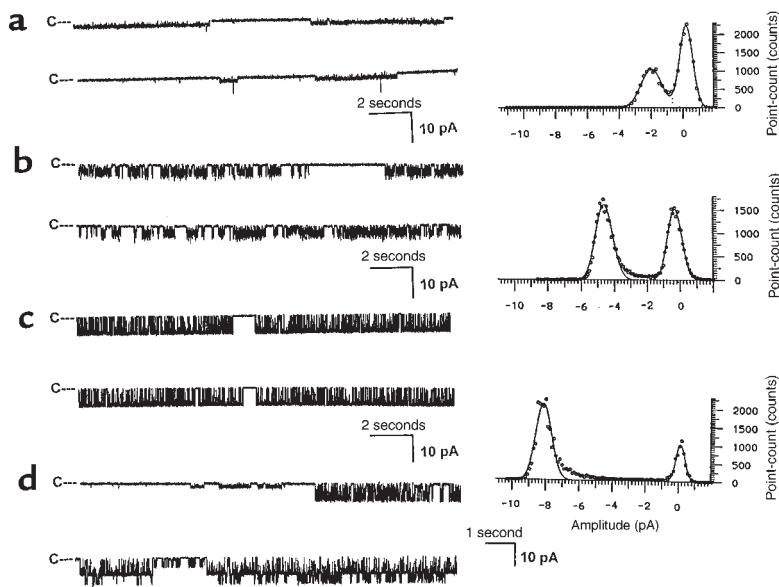
O<sub>2</sub> is an essential requirement for cell survival in mammals. When a cell, and in particular a neuron, lacks O<sub>2</sub> in its microenvironment, various sets of mechanisms are activated depending on the severity and duration of O<sub>2</sub> deprivation, as well as on other factors such as the developmental stage and differentiation of the cells, their O<sub>2</sub> consumption, and their genetic endowment (1). For example, one set of mechanisms is ionic in nature, and this has been shown to be crucial in nerve cell injury or survival. In particular, Na<sup>+</sup> and K<sup>+</sup> channels have been implicated in a number of pathways that are important for cell adaptation or injury (1–4).

K<sup>+</sup> channels have been shown to play a key role in adaptive mechanisms in response to hypoxia and are thought to be involved in sensing O<sub>2</sub> deprivation. Regulation of K<sup>+</sup> channels by O<sub>2</sub> tension has been demonstrated in rabbit type I carotid body cells (5), pulmonary vascular smooth muscle cells (6), airway neuroepithelial bodies (7), and central neurons (8). Furthermore, various types of O<sub>2</sub>-regulated K<sup>+</sup> conduc-

tances have been demonstrated, such as the delayed rectifier K<sup>+</sup> channel (7, 9, 10), ATP-dependent K<sup>+</sup> channel (8), and Ca<sup>2+</sup>-activated K<sup>+</sup> channel (11).

Ca<sup>2+</sup>-activated K<sup>+</sup> channels (K<sub>Ca</sub>) have been identified in various cell types, including central neurons, and are generally distinguished from other channels by their single-channel conductance, Ca<sup>2+</sup> sensitivity, voltage dependence, and unique pharmacological properties (12). All K<sub>Ca</sub> channels are activated by an increase in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>), and some of them can also be modulated by other messenger systems (13–15). From a physiological point of view, K<sub>Ca</sub> channels are particularly interesting because they may provide a link between second messenger systems and membrane conductance. Hypoxia has been shown to induce inhibition of K<sub>Ca</sub> channels in cells from carotid body (11, 16), pulmonary smooth muscle (17), and the adrenal gland (18). However, the mechanisms underlying this inhibition are not well understood.

Neurons in the mammalian central nervous system (CNS) are known to be highly sensitive to the avail-



**Figure 1**

Different types of  $K^+$  channels in isolated neocortical neurons of mice. Currents were recorded from inside-out patches of a neocortical neuron using symmetrical 140 mM KCl on both sides of the membrane at a  $V_m$  of  $-40$  mV. The channel closed level is indicated by "C". (a) Example traces of small-conductance  $K^+$  channel. The amplitude histogram to the left was compiled from 40 seconds of current record digitized at 500 Hz. (b) Example traces and the amplitude histogram of the medium-conductance  $K^+$  channel. (c) Example traces and the amplitude histogram of the large-conductance  $K^+$  channel. (d) Example traces containing multiple  $K^+$  channel types.

ability of  $O_2$ . A number of investigators, including ourselves, have shown that  $O_2$  deprivation depolarizes adult central neurons to various degrees (2, 19, 20). The mechanisms underlying this change in membrane potential and consequently in ionic activities and currents are not clear, although we and others have demonstrated the role of some ionic mechanisms (20, 21). Given that  $K^+$  channels are known to play a key role in the maintenance of membrane potential and in neuronal excitability, it is possible that the depolarization during hypoxia is mediated via inactivation of certain  $K^+$  channels. We hypothesized therefore that  $K_{Ca}$  channels, an important channel in neurons, are involved in the response of the CNS to reduced microenvironmental  $O_2$ . To test our hypothesis, we studied the effect of hypoxia on the  $K_{Ca}$  channels in neocortical neurons of mice. Furthermore, we examined potential underlying mechanisms that play a role in modulating  $K_{Ca}$  channels during hypoxia. This work has an added significance, as the regulation of this channel's activities may be important for therapeutic interventions aimed at reducing neuronal excitability during hypoxic or ischemic stress in the CNS.

## Methods

**Cell isolation.** Neurons were harvested from the temporal cortex of mice (25–30 days old) using a modified method of Kay and Wong (22). In brief, mice were deeply anesthetized with methoxyflurane and decapitated. The brain was removed rapidly, chilled in  $0$ – $4^\circ$  C Ringer's solution, and prepared as a tissue block. The tissue block was sectioned transversely into 300- $\mu$ m slices at the level immediately above the pons, and 2 or 3 slices were taken. Slices were incubated for 30 minutes with oxygenated HEPES buffer containing (in mM) 140 NaCl, 2.5 KCl, 1  $MgCl_2$ , 1  $CaCl_2$ , 10 D-glucose, 10 HEPES, and trypsin (0.02–0.04%, type XI; Sigma Chemical Co., St. Louis, Missouri, USA). After

exposure to trypsin, slices were washed and incubated for 20 minutes with Protease (0.01–0.02%, type XIV; bacterial; Sigma Chemical Co.). Slices were then washed in oxygenated HEPES buffer and maintained for up to 6 hours.

Immediately before recording, individual tissue slices were removed and placed in oxygenated HEPES buffer. Using a dissecting microscope, the gray matter (including layers 3–5 of neocortex) were cut free from the rest of the slice. The tissue of interest was then dissociated by gentle trituration with fire-polished Pasteur pipettes. Cells were plated in 35-mm Petri dishes and observed with Hoffman modulation optics. Recordings were obtained only from pyramidal-shaped neurons with a single thick proximal dendrite (presumably pyramidal neurons) and did not show visible evidence of injury. Flat or swollen cells or cells with a grainy membrane appearance were not studied as described previously (22, 23).

**Patch-clamp techniques.** Patch-clamp experiments were performed at room temperature ( $\sim 24^\circ$  C). Single-channel currents were recorded from cell-attached and excised membrane patches using standard patch-clamp recording procedures (24). Recording pipettes were made from 1.2-mm borosilicate capillary glass using a Sutter P-84 puller (Sutter Instrument Co., Novato, California, USA). The pipettes were fire-polished and had resistances of 8–15  $M\Omega$  when filled with 140 mM KCl. Current recordings were obtained using an Axopatch 200A amplifier (Axon Instruments, Foster City, California, USA) and recorded into a digital audio tape. The tape was later replayed through an 8-pole Bessel filter ( $-3$  dB at 1 kHz) and digitized at 5 kHz with a 12-bit analog-to-digital converter (Neuro-Corder model DR-484, Neuro Data Instruments Corp., New York, New York, USA). The data were analyzed with the use of the pCLAMP software system 6.02 (Axon Instruments, Burlingame, California, USA).

$NP_o$  was determined from data samples of 1-minute duration and defined as:  $NP_o = \sum (t_1 + 2t_2 + 3t_3 + \dots + nt_n)$ , where  $N$  is channel number,  $P_o$  is open probability, and  $t_1, t_2, t_n$  are the ratios of open time to total time of measurement for each channel at each of the current levels. The mean values of unitary current levels were determined by fitting the amplitude histogram with Gaussian curve.

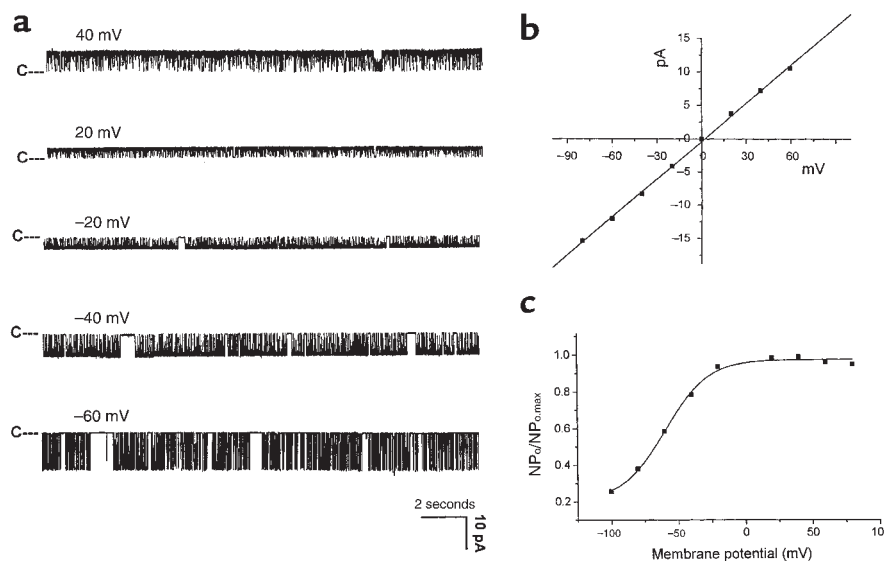
**Experimental solutions and reagents.** The pipette solution contained (in mM) 140 KCl, 1 MgCl<sub>2</sub>, 1 EGTA, 10 D-glucose, and 10 HEPES (pH 7.4). The bath solution for cell-attached patches was composed of (in mM) 140 NaCl, 2.5 KCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 10 D-glucose, 10 HEPES (pH 7.4). Under these ionic conditions, the resting potential of neocortical neurons was measured as  $-106 \pm 5.4$  mV ( $n = 11$ ). The composition of the bath solution for inside-out patches was the same as that of the pipette solution except for CaCl<sub>2</sub>. The concentrations of free Ca<sup>2+</sup> and Mg<sup>2+</sup> were calculated using CAMG software (Yale University, New Haven, Connecticut, USA). Hypoxia was induced by bubbling perfusing solutions with argon for at least 2 hours. The O<sub>2</sub> tension, PO<sub>2</sub>, was measured with polarographic electrodes (8 μm in diameter) (25) and ranged between 10 and 20 mmHg. All chemicals were purchased from Sigma Chemical Co., except charybdotoxin (ChTX) and iberiotoxin (IbTX), which were obtained from Research Biochemicals International (Natick, Massachusetts, USA), and dendrotoxin-I (DTX-I), which was obtained from Alomone Labs Ltd. (Jerusalem, Israel). Native ChTX was also purified from *Leiurus* scorpion venom as described (26).

**Analysis and statistics.** Data are shown as mean  $\pm$  SEM, and paired Student's *t* test was used to determine the significance between control and experimental periods. Means were considered significantly different from each other when  $P < 0.05$ .

## Results

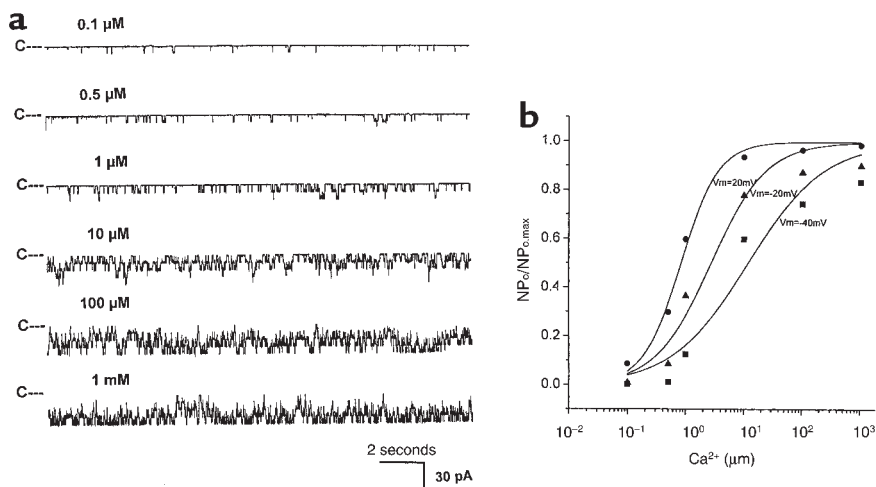
**Characterization of single Ca<sup>2+</sup>-activated K<sup>+</sup> channels in mice neocortical neurons.** Cell-attached and excised membrane patches of neocortical neurons contained several types of ionic channels. Among them, three types of K<sup>+</sup> channels with different conductances were detected under ionic conditions consisting of 140 mM KCl (i.e., no Na<sup>+</sup> and low Ca<sup>2+</sup>) in both bath and pipette solutions (Figure 1). In particular, a large-conductance channel was observed in the majority of patches (>70%). In addition to this large-amplitude current, 2 other smaller unitary K<sup>+</sup> channel currents could also be observed in some patches. They were of medium and small amplitude: under symmetrical 140 mM KCl, their single-channel conductances were  $118 \pm 8.9$  pS ( $n = 8$ ) and  $54 \pm 5.8$  pS ( $n = 14$ ), respectively. Further, these 2 smaller conductances did not exhibit any sensitivity to intracellular Ca<sup>2+</sup> (Ca<sup>2+</sup><sub>i</sub>) and could not be modulated by O<sub>2</sub> tension in both cell-attached and excised patches. Thus, these were not investigated further in this study.

Under symmetrical 140 mM KCl, the large-conductance channels displayed a linear current-voltage relationship with a mean single-channel conductance of  $210 \pm 6.9$  pS ( $n = 15$ ) (Figure 2, a and b). Under approximate physiological conditions (i.e., 140 mM NaCl and 2.5 mM KCl in the pipettes and 140 mM KCl in the bath), the reversal potential was approximately  $-110$  mV, close to the calculated value from the Nernst equation, assuming that the charge carrier is K<sup>+</sup>. This large-conductance K<sup>+</sup> channel was activated by membrane depolarization and elevation of Ca<sup>2+</sup> on the cytosolic side of excised patches and exhibited sustained noninactivating activity in the presence of a fixed voltage and cytoplasmic Ca<sup>2+</sup> concentration. At more negative potentials, there were a few channel openings of short duration, but with increasing membrane depolarization, the open probability increased (Figure 2c). The open probability was also dependent on free Ca<sup>2+</sup> on



**Figure 2**

(a) Single large-conductance K<sup>+</sup> currents recorded from an inside-out patch, recorded at various holding potentials in the presence of symmetrical 140 mM KCl across the neuronal membrane. (b) The current-voltage relationship of the large-conductance K<sup>+</sup> channel under conditions already described. The line drawn through these points is a linear regression fit with a conductance of  $210 \pm 6.9$  pS ( $n = 15$ ). (c) Relationship between open probability and voltage for this large-conductance channel. The line is fitted to a Boltzmann distribution:  $y = 1 / \{1 + \exp [(V_{0.5} - V_m) / k]\}$ , where  $y$  is normalized open probability ( $NP_o / NP_{o,max}$ ),  $V_m$  is membrane potential,  $V_{0.5}$  is the membrane potential at which  $NP_o$  is half of  $NP_{o,max}$ , and  $k$  is a constant related to the slope of curve.  $V_{0.5} = -46$  mV.



**Figure 3**

(a) Single-channel recordings of BK<sub>Ca</sub> channel activity in the presence of varying concentration of Ca<sup>2+</sup>; at a V<sub>m</sub> of -40 mV. (b) Relationship between Ca<sup>2+</sup> and channel open probability at various membrane potentials. Data were fitted to Hill's equation:  $y = 1 / \{1 + ([Ca^{2+}]_i/k)^h\}$ , where  $y$  is the normalized open probability (NP<sub>o</sub>/NP<sub>o,max</sub>), [Ca<sup>2+</sup>]<sub>i</sub> is intracellular free Ca<sup>2+</sup> concentration,  $k$  is the free Ca<sup>2+</sup> concentration required for maximum channel activation, and  $h$  is the Hill coefficient.

the cytosolic side of the patches (Figure 3a), and this dependence was also examined at different membrane potentials. At each voltage, a Ca<sup>2+</sup> activation curve was fitted to Hill's equation, as seen in Figure 3b. This shows that the half free Ca<sup>2+</sup> concentration for maximum channel activation ( $k$ ) was increased from 0.96 to 3.04 μM and to 12.09 μM, when the membrane potential was changed from 20 to -20 mV and to -40 mV, respectively. An increase in Ca<sup>2+</sup> from 1 to 2 mM produced only a small increase in open probability, indicating that the Ca<sup>2+</sup> gating sites are saturated at 1 mM and the channel is fully activated. When the cytosolic side of excised patches was perfused with a solution containing nominally no Ca<sup>2+</sup> and 2 mM EGTA, no channel opening was observed at voltages between -100 and 100 mV. Therefore, based on the criteria of unitary conductance, ionic selectivity, and synergistic action by voltage and Ca<sup>2+</sup>, this channel may be identified as a maxi K<sub>Ca</sub> or BK<sub>Ca</sub> channel (27).

We also examined the sensitivity to different K<sup>+</sup> channel blockers. Although tetraethylammonium chloride (TEA, 1 mM) did not block the BK<sub>Ca</sub> currents when it was applied to the internal side of patches ( $n = 3$ ), TEA at the same dose was found to block BK<sub>Ca</sub> in outside-out patches ( $n = 5$ ) by suppressing the current amplitude. Figure 4a shows the effect of increasing the concentration of TEA on the external side of a single BK<sub>Ca</sub> channel from 0 to 1 mM, and the progressive decrease in amplitude of the unitary current was observed as a function of TEA. When the apparent single-channel current is normalized to the unblocked current and plotted as a function of external TEA concentration, the  $K_d$  obtained was  $0.42 \pm 0.04$  mM ( $n = 5$ ). Although some BK<sub>Ca</sub> channels are known to be sensitive to the nanomolar concentration of external ChTX, a brain ChTX-insensitive BK<sub>Ca</sub> channel has also been reported (13, 28). Interestingly, in this present study, there was no significant blocking action of ChTX (100 nM–1 μM) or IbTX (20–100 nM) on this BK<sub>Ca</sub> channel in both inside-out ( $n = 3$ ) and outside-out patches ( $n = 9$ ) (Figure 4b). The mamba snake neurotoxin, DTX-I, has been previously

described to induce a long-lived subconductance state from the internal side in BK<sub>Ca</sub> channels from rat skeletal muscle incorporated into planar bilayers (29). In the present study, DTX-I (200 nM) also induced the appearance of distinct subconductance events with 72% of the normal open state current at -40 mV when present on the internal side of BK<sub>Ca</sub> channels ( $n = 4$ ) (Figure 4c). This observation supports the idea that the ChTX-insensitive type of BK<sub>Ca</sub> channels studied here are structurally related to ChTX-sensitive BK<sub>Ca</sub> channels, as they both share a common sensitivity to internal DTX-I.

*Effect of hypoxia on single-channel BK<sub>Ca</sub> current.* Previous studies have shown that BK<sub>Ca</sub> channels, isolated from rabbit pulmonary artery smooth muscle cells (17) and carotid body cells (11) are downregulated by hypoxia. Other studies, however, have shown that reduced O<sub>2</sub> tension increases the activity of BK<sub>Ca</sub> in cat cerebral arterial smooth muscle cells (30). We examined in this work the effect of hypoxia on the BK<sub>Ca</sub> channel in neocortical neurons. Single BK<sub>Ca</sub> channels were recorded in cell-attached patches of isolated neocortical neurons. With high-KCl (140 mM) solution in the pipette and physiological solution in the bath, a low-O<sub>2</sub> medium (PO<sub>2</sub> = 10–20 mmHg) decreased single-channel activity markedly after a latency of 5–7 minutes (Figure 5, a and c). This inhibitory effect of low PO<sub>2</sub> was observed in 9 of 14 experiments and was characterized by a decrease in open probability without significantly affecting single-channel current amplitude or the unitary slope conductance in the membrane potential range of -60 mV to +40 mV. Furthermore, the hypoxic inhibition of BK<sub>Ca</sub> channel activity is strongly voltage dependent. At -30 mV, NP<sub>o</sub> was markedly reduced to  $43.4 \pm 4.3\%$  of control levels after exposure to hypoxia for 10 minutes ( $n = 9$ ). But at +20 mV, NP<sub>o</sub> was reduced to only  $84.5 \pm 4.8\%$  of control levels. Data for the variation in channel open probability with membrane potential was fitted to the Boltzmann equation, which allowed the estimates of the half-activation voltage (V<sub>0.5</sub>). We found that the mean V<sub>0.5</sub> shifted to more depolarized potential after exposure of hypoxia (Figure

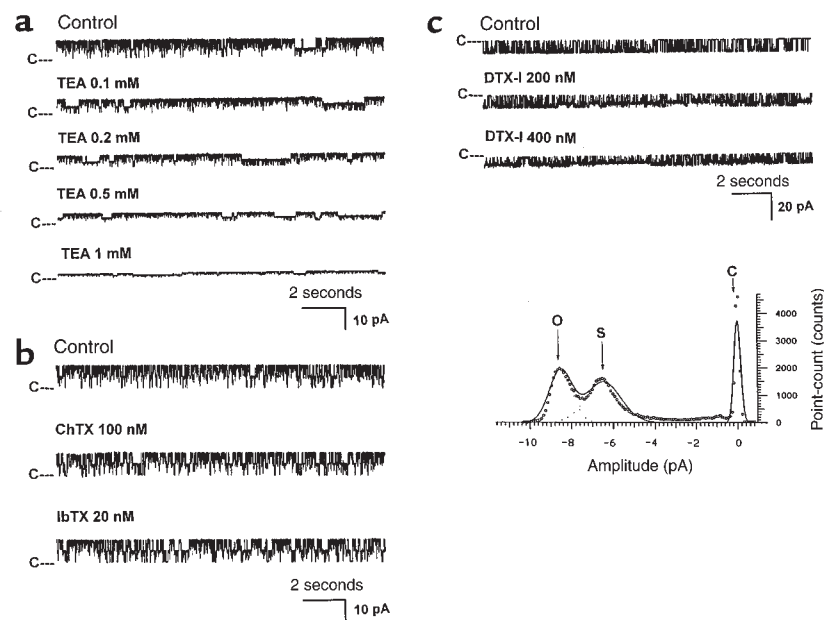
5b). Reperfusion with oxygenated solution led to partial or complete recovery in most patches. On average,  $NP_o$  recovered to  $77.9 \pm 6.8\%$  ( $n = 9$ ) of baseline level, 5–10 minutes after reoxygenation. Taken together, low  $PO_2$  inhibited  $BK_{Ca}$  channel activity when the membrane potential was negative or slightly depolarized, and the inhibition was attenuated if the membrane was depolarized to potential more positive than +20 mV. This suggests that hypoxia-induced inhibition of  $BK_{Ca}$  channel activity may have greater physiological significance at more negative membrane potentials in the neuronal response to hypoxia. To determine whether this hypoxia-induced reduction in  $BK_{Ca}$  channel activity is a direct or indirect effect of  $O_2$  deficiency, we next examined the effect of hypoxia on  $BK_{Ca}$  channels in inside-out patches. We found that neither single-channel open probability nor amplitude was significantly affected by hypoxia (Figure 5d). These results suggested that hypoxic inhibition of  $BK_{Ca}$  channel is not determined by a closely coupled, membrane-limited mechanism. Instead, it appears that cytosolic alterations are required for hypoxia-mediated events.

Having established that  $O_2$  deprivation inhibited  $BK_{Ca}$  channel via cytosol-dependent processes, we next examined the effects of possible cytosolic changes on channel activity during hypoxia. Hypoxia has been shown, for example, to decrease intracellular pH ( $pH_i$ ), increase  $Ca^{2+}$ , increase the activity of kinases, and alter the energy charge and redox potential in cells (1). In this work, we examine some of these factors and determine their effect on  $BK_{Ca}$ .

*The effect of pH on  $BK_{Ca}$  channel activity.* The concentration of  $H^+$  present at the intracellular surface has been previously shown to be an important modulator of  $BK_{Ca}$  channel activity in many tissues (31–33). In this regard, we have made several observations with respect

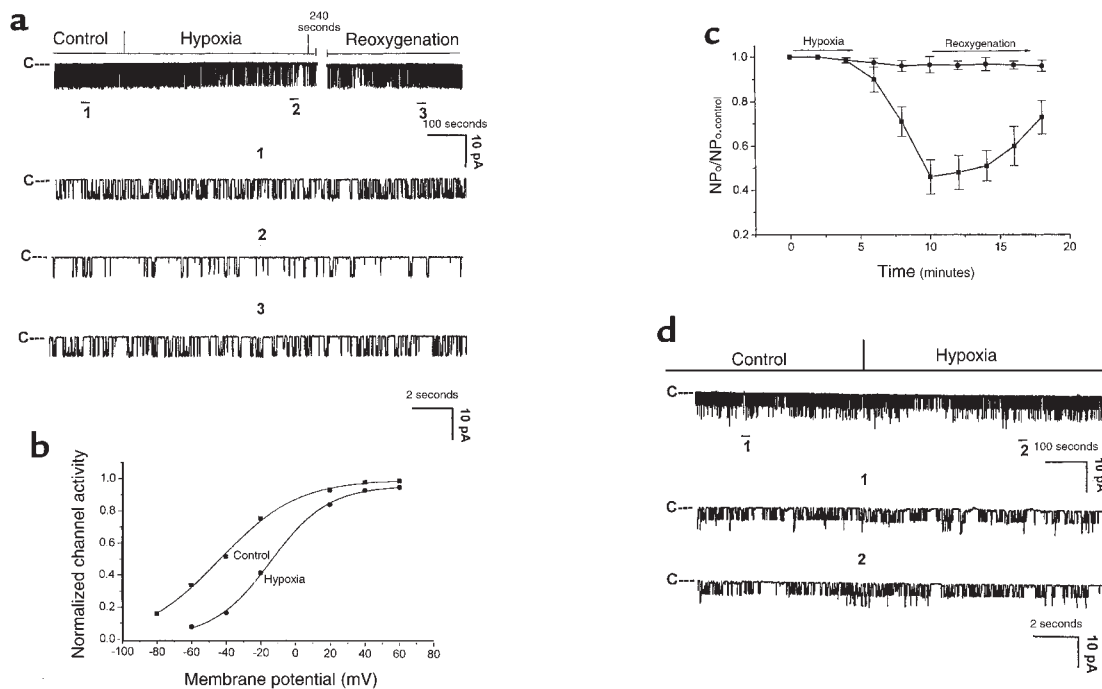
to pH and this channel activity. At a concentration of  $100 \mu M Ca^{2+}$  and a membrane potential of  $-40$  mV, a reduction in pH on the cytosolic side from 8.0 to 7.0 did not change the open probability of the channel significantly. However, channel activity was reduced dramatically when pH was changed from 6.4 to 5.6 (Figure 6). At a pH of 6.0 and 5.6, channel  $NP_o$  at  $-30$  mV was reduced to  $12.2 \pm 3.1\%$  ( $n = 7$ ) and  $5.9 \pm 0.82\%$  ( $n = 7$ ) of control level, respectively. In addition to change in channel  $NP_o$ , lowering cytosolic pH increased single-channel current amplitude. At pH 5.6 and a membrane potential of  $-30$  mV,  $BK_{Ca}$  channel exhibited a single-channel current amplitude of  $7.78 \pm 0.46$  pA compared with the control level of  $6.25 \pm 0.27$  pA at pH 7.2 ( $n = 7$ ;  $P < 0.05$ ). The  $H^+$  inhibition was fully reversed by returning the pH to neutral (Figure 7a), demonstrating that the channel had not been denatured by exposure to acidic pH. Figure 7b showed that even a total block of the  $K^+$  channel activity by acidification could be partially lifted by the addition of high concentration of  $Ca^{2+}$  onto the cytoplasmic side of the channels. The inhibitory effect of  $H^+$  suggests that protons decrease the sensitivity of  $K^+$  channel to  $Ca^{2+}$ . We have therefore examined the effect of pH on  $Ca^{2+}$  activation of the channel. Fitting the data to Hill's equation at pH 6.0 and a membrane potential of  $-40$  mV, the  $Ca^{2+}$  activation curve was shifted to the right when compared with the activation curve at pH 7.2 (Figure 7d). The  $Ca^{2+}$  concentrations responsible for half-maximum channel activation was  $12.09 \mu M$  and  $1.92$  mM at pH 7.2 and 6.0, respectively. The effect of lowering the pH had a similar effect as rendering the membrane potential more negative, with the  $K^+$  channel becoming less sensitive to  $Ca^{2+}$ .

*Effect of ATP and phosphorylation upon  $BK_{Ca}$  channel.* Other workers have shown that  $BK_{Ca}$  channel, isolated from rat brain, can be regulated by protein kinase A



**Figure 4**

(a) Currents recorded under symmetrical 140 mM KCl condition in an outside-out patch from a neocortical neuron at a  $V_m$  of 30 mV. TEA (0–1 mM), from the extracellular side, blocks the  $BK_{Ca}$  channel. (b) Effects of ChTX (100 nM) and IbTX (20 nM), also on the extracellular side of the  $BK_{Ca}$  channels. Currents were also recorded in an outside-out patch at a  $V_m$  of  $-40$  mV. DTX-I (200–400 nM) induced a subconductance state with 72% of normal open-state current. Amplitude histogram to the upper trace was compiled from 60 seconds (DTX-I 200 nM) of current record digitized at 500 Hz. Amplitude peaks are identified as closed state (c), substate (s), or open state (o).



**Figure 5**

(a) Effect of hypoxia on single BK<sub>Ca</sub> channel in a cell-attached patch from a neocortical neuron. Current was recorded with high-KCl (140 mM) solution in the pipette and physiological solution in the bath at a V<sub>m</sub> of -30 mV. The channel closed level is indicated by “C”. Three parts of the compressed trace are shown as indicated by numbers 1–3 (fast time resolution). (b) Effect of hypoxia on the voltage activation of BK<sub>Ca</sub> channel under ionic condition just described. The line is fitted to a Boltzmann distribution. V<sub>0.5</sub> shifted from -42.4 ± 4.8 mV to -18.6 ± 3.5 mV after exposure of hypoxia for 10 minutes. (c) Time course of the hypoxia-induced effect on NP<sub>0</sub>. In cell-attached recordings (filled squares), channel inhibition started about 5 minutes after the onset of hypoxia, and a maximum inhibition was reached in about 10 minutes. After that time, NP<sub>0</sub> was markedly reduced to about 43% of control level. Reoxygenation led to partial recovery. In inside-out recordings (filled circles), NP<sub>0</sub> was not significantly affected during hypoxia. (d) Continuous recording of a single BK<sub>Ca</sub> channel current from an inside-out patch of a neocortical neuron during hypoxia, using a symmetrical 140 mM KCl on both sides of the membrane, with a V<sub>m</sub> of -30 mV. The channel closed level is indicated by “C”. Two parts of the compressed trace (indicated by the numbers 1 and 2) are shown below at fast time resolution.

(PKA) (13). Furthermore, it has been demonstrated that at least 1 type of neuronal BK<sub>Ca</sub> can be activated by the intracellular application of ATP (33). Therefore, we tested the effect of cytosolic ATP and phosphorylation on BK<sub>Ca</sub> channel activity in inside-out patches, with 100 μM Ca<sup>2+</sup> in the bath solution. Addition of 1 mM to 3 mM Mg-ATP had no significant effect on both open probability and single-channel conductance, if Ca<sup>2+</sup> and pH were tightly controlled. Glibenclamide (10–100 μM) also did not block this BK<sub>Ca</sub> channel activity. However, the addition of the PKA catalytic subunit (20 U/mL) to the bath solution in the presence of 1 mM Mg-ATP caused a decrease of open probability to 52.3 ± 3.5% of control (*n* = 6) (Figure 8a). On the other hand, heat-inactivated PKA had no effect on the channel activity. In addition to the change in NP<sub>0</sub>, PKA also induced a significant reduction in single-channel amplitude, the BK<sub>Ca</sub> channel exhibited a single-channel current amplitude of 4.54 ± 0.22 pA at -30 mV, compared with 6.25 ± 0.13 pA for control (*n* = 6). Interestingly, although a reduction in pH to 7.0 on the cytosolic side did not change channel activity significantly, PKA-induced inhibition was markedly enhanced at pH 7.0, with the open probability reduced to 32.8 ± 5.2% of

control (*n* = 3) (Figure 8c). The PKA inhibition could be fully reversed by washout, demonstrating that the channel had not been denatured by PKA. Moreover, we found that the inhibiting effect of BK<sub>Ca</sub> by PKA could be partially lifted by the addition of high concentration of Ca<sup>2+</sup> (5 mM) (Figure 8b), suggesting that PKA, like pH, decreases the sensitivity of this K<sup>+</sup> channel to Ca<sup>2+</sup> in a fashion similar to that of pH. We therefore examined the effect of PKA on the Ca<sup>2+</sup> activation of the channel in the presence of PKA. PKA (20 U/mL) caused a major change in the Ca<sup>2+</sup> concentration required for half-maximum channel activation, e.g., from 12.09 to 96.8 μM at membrane potential of -40 mV, with a shift of the curve to the right (*n* = 4) (Figure 8d).

To examine whether a similar system might regulate BK<sub>Ca</sub> channel activity in intact neocortical cells, we also studied the effect of PKA activator on these channels in the cell-attached configuration. Application of 0.1 mM membrane permeable cAMP analogue dibutyryl cAMP (db cAMP) inhibited BK<sub>Ca</sub> channel activity, decreasing NP<sub>0</sub> to 59.6 ± 4.8% (*n* = 5) of control levels (Figure 9, a and c). The inhibition was slow to develop but was sustained even after db cAMP removal from the bath solution. Given that it has been reported that PKC could

modulate BK<sub>Ca</sub> channel through phosphorylation (34), and because we have evidence from our previous work that protein kinase C (PKC) is activated during hypoxia (3), we studied the effect of PKC on BK<sub>Ca</sub> channel activity. However, the addition of 100–500 nM PMA, a potent PKC activator, did not show any effect on BK<sub>Ca</sub> channel activity (Figure 9b).

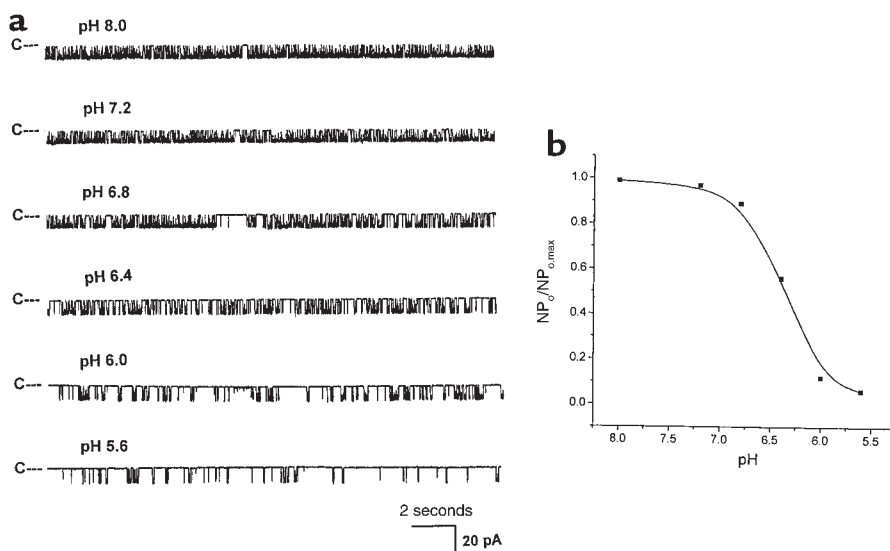
**Effects of redox agents on BK<sub>Ca</sub> channel activity.** Modulation of ion channels by cellular redox potential has emerged recently as a significant determinant of channel function (35). One major endogenous redox factor in the brain that may control BK<sub>Ca</sub> channel function in the intact cell is glutathione(GSH), present in millimolar concentration (36). The majority of glutathione located within cells is in the reduced form. During periods of oxidative stress, GSH is converted to the oxidized form (GSSG) (37). Therefore the influence of the cytosolic redox agents on the channel activity was first investigated by testing the effect of the reducing agent GSH and the oxidizing agent GSSG. Single BK<sub>Ca</sub> channels were recorded from inside-out patches of isolated neocortical neurons; 1 mM GSH markedly increased the single BK<sub>Ca</sub> channel activity (Figure 10a). This stimulatory effect of GSH was characterized by an increase in open probability without significantly affecting single-channel current amplitude or the unitary slope conductance when the holding potential and pH were tightly controlled. At -30 mV, NP<sub>o</sub> was markedly increased to 146.4 ± 6.3% of control level after addition of 1 mM GSH (*n* = 4). The augmenting effect was generally observed after about 1 minute of GSH application and was maintained despite the removal of GSH from the bath. Conversely, application of 1–5 mM GSH (a relatively membrane-impermeant reducing agent) to the extracellular surface in outside-out patches had no significant effect on channel activity (*n* = 2; data not shown), which suggested that redox modulation occurs at the cytosolic surface of neocortical neurons. In contrast to GSH, 1–5 mM GSSG had no apparent effect

(Figure 10b). However, the maintained increase in channel activity caused by GSH recovered to control level with the addition of GSSG (Figure 10c), indicating that the change in channel activity after GSH application resulted from redox state modification rather than being due to a nonspecific effect of this compound. To support this idea further, we next examined the effect of redox-based chemicals such as DTT and DTNB on BK<sub>Ca</sub> channel activity. As shown in Figure 10, d and e, the reducing agent DTT (1 mM) markedly augmented BK<sub>Ca</sub> channel activity in inside-out patches, and this was reversed by the oxidizing agent DTNB (1 mM). Like GSSG, DTNB (1–5 mM) alone had no significant effect on BK<sub>Ca</sub> channel. Taken together, these results provide evidence that BK<sub>Ca</sub> channel in mice neocortical neurons can be modulated by a change in the cellular redox potential, serving to link the metabolic state to the electrical activity of the neuron.

## Discussion

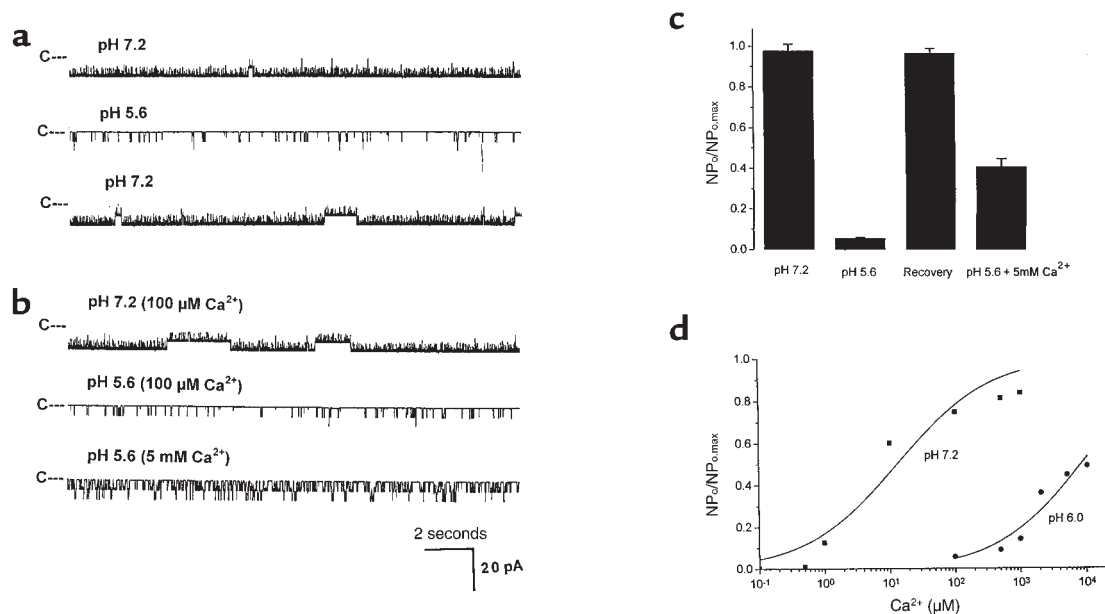
It is well recognized that the consequences of a hypoxic or ischemic episode in neurons are not only determined by the severity and duration of O<sub>2</sub> and nutrient deprivation, but they are also influenced by the cellular and membrane properties of these neurons. In mammalian central neurons, cells that are extremely vulnerable to O<sub>2</sub> deprivation, 1 of the hypoxia-induced important functional alterations is membrane depolarization. Although we have elucidated some of the mechanisms responsible for this hypoxia-induced depolarization in the past, we believe that such mechanisms are still not all well understood. For example, in spite of the fact that removal of extracellular Na<sup>+</sup> attenuates this hypoxia-induced depolarization, it does not eliminate it (21). Moreover, we do not know what the mechanism is for Na<sup>+</sup> influx under hypoxic conditions.

K<sup>+</sup> channels are known to play a key role in the maintenance of membrane potential and in neuronal activity. We have also shown in our previous work that there are



**Figure 6**

Cytosolic pH modulates NP<sub>o</sub> of BK<sub>Ca</sub> channels in inside-out membrane patches from neocortical neurons. (a) Example from a patch. Currents were recorded at a V<sub>m</sub> of -40 mV. Changing the pH of the bath solution (intracellular side of the membrane) leads to marked changes in NP<sub>o</sub>. (b) Relationship between pH<sub>i</sub> and channel NP<sub>o</sub> at a V<sub>m</sub> of -40 mV. Data were fitted to Hill's equation. The half-inhibition pH value was 6.42.



**Figure 7** Reversibility of the pH effect on the BK<sub>Ca</sub> channels. Currents were recorded from an inside-out patch of a neocortical neuron, using symmetrical 140 mM KCl on both sides of the membrane, V<sub>m</sub> of -30 mV. (a) Single-channel trace showing inhibition by pH 5.6 and complete reactivation at pH 7.2. (b) Similar experiment, but where the reactivation was done by the addition of 5 mM Ca<sup>2+</sup>. (c) Mean change of NP<sub>0</sub>, measured at different conditions. (d) Effect of pH (6.0 and 7.2) on the relation of Ca<sup>2+</sup> and NP<sub>0</sub>. Data were acquired from inside-out patches of neocortical neurons using symmetrical 140 mM KCl on both sides of the membrane, V<sub>m</sub> of -40 mV, and fitted to Hill's equation.

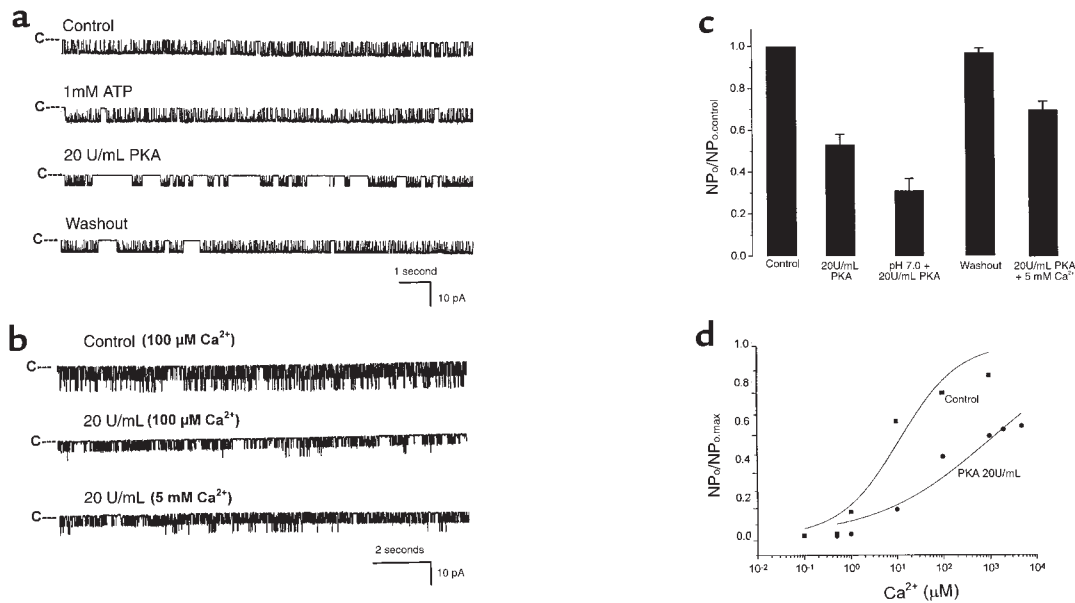
major alteration in K<sup>+</sup> ions homeostasis during hypoxia (38). It would then be reasonable to expect that the regulation of K<sup>+</sup> channel activity by hypoxia might have a major impact on the membrane potential as well as neuronal function. In this study, we have examined the effects of O<sub>2</sub> deprivation on K<sup>+</sup> channel activity, recorded from freshly isolated neocortical neurons of mice. Under symmetrical K<sup>+</sup> conditions, 3 main types of K<sup>+</sup> channels were identified in our patches. Two, rather small, K<sup>+</sup> channel conductances were observed in cell-attached patches. However, these did not show any sensitivity to hypoxia and were hence excluded from this study. We focused our study on the third conductance, which was sensitive to lack of O<sub>2</sub> and was very frequently seen (see Results). In the presence of symmetrical 140 mM KCl with a bath Ca<sup>2+</sup> of 200 μM, the single-channel conductance of this K<sup>+</sup> channel was 210 pS. Furthermore, this channel exhibited strong voltage-dependent activation and a marked sensitivity to Ca<sup>2+</sup>. Although ChTX and IbTX did not block this channel, because of its unitary conductance, ionic selectivity, and synergistic action by voltage and Ca<sup>2+</sup>, and other unique pharmacologic properties, we believe that this is a ChTX-insensitive Ca<sup>2+</sup>-activated K<sup>+</sup> channel (BK<sub>Ca</sub> channel), similar to type II BK<sub>Ca</sub> channels previously identified in rat brain (13).

Because of the effect of hypoxia in the cell-attached patches and the absence of an effect in inside-out patches, it would seem likely that this hypoxia-induced inhibition of BK<sub>Ca</sub> channel is mediated by cytosolic factors. Clearly, hypoxia, if severe enough, is usually accompanied by compensatory or pathophysiologic

changes that could alter channel function and sensitivity. For example, hypoxia can produce a number of cytosolic changes that we and others have demonstrated in the past such as an increase in cytosolic Ca<sup>2+</sup> concentration and a drop in pH (1). The question is raised, however, as to what effects do all of these factors have on this BK<sub>Ca</sub> individually and when they are all operating together. For instance, an increase in Ca<sup>2+</sup><sub>i</sub> would be expected to activate the BK<sub>Ca</sub> channel; however, we found that BK<sub>Ca</sub> decreased its activity during hypoxia. Therefore, this situation can be very complex, as these various factors that affect channel activity occur at different time after instituting hypoxia, have different time constants, and may have interactive effects on the channel such as pH and PKA. Furthermore, the BK<sub>Ca</sub> channel may have different sensitivities to factors such as Ca<sup>2+</sup> during hypoxia, or the local concentration of Ca<sup>2+</sup><sub>i</sub>, in the vicinity of BK<sub>Ca</sub> channel, may increase more slowly than elsewhere in the cell. In addition, little is known regarding possible increase in the local extracellular K<sup>+</sup> concentration owing to the opening of BK<sub>Ca</sub> channels. Because of the potential complexity of the situation during hypoxia, it is imperative then to dissect the role of various alterations separately. At present, some of the well-known alterations include intracellular concentration of proton and nucleotides, protein phosphorylation and proteolytic enzyme activity, redox activity, and receptor modulation.

*pH<sub>i</sub> and BK<sub>Ca</sub> channel activity.* Previous studies have shown that hypoxia induces intracellular acidification in neurons (39, 40). A variety of ion channels can be





**Figure 8**

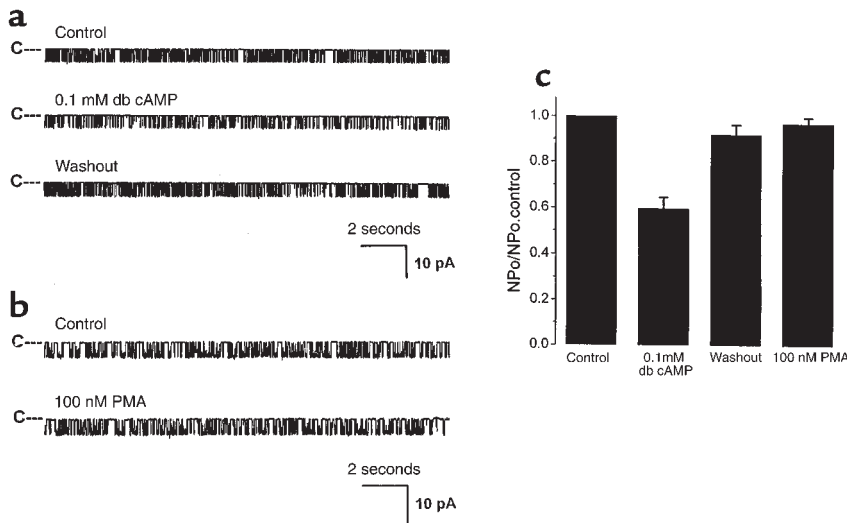
Effect of the catalytic subunit of PKA on the BK<sub>Ca</sub> channel. Currents were recorded from inside-out patches of neocortical neurons, using symmetrical 140 mM KCl on both sides of the membrane, V<sub>m</sub> of -30 mV. (a) Single-channel traces showing the inhibition by PKA 20 U/mL and reactivation upon washing out. (b) Similar experiment, where the reactivation was done by the addition of 5 mM Ca<sup>2+</sup>. (c) Mean change in NP<sub>0</sub>, measured at different conditions. (d) Effect of 20 U/mL PKA on the relation between Ca<sup>2+</sup> and NP<sub>0</sub>. Data were acquired from inside-out patches of neocortical neurons using symmetrical 140 mM KCl on both sides of the membrane, V<sub>m</sub> of -40mV and fitted to Hill's equation.

influenced by modest shifts in H<sup>+</sup> and might thereby modulate neuronal excitability (41). Intracellular acidification has been shown to cause depolarization in mammalian neurons and glial cells (42, 43), and it is possible therefore that this is mediated by inactivation of certain K<sup>+</sup> channels. In the present study, changes in pH<sub>i</sub> produced 2 effects on BK<sub>Ca</sub> channel in mice neocortical neurons, mainly on channel activity but also on single channel conductance. Our data showing that intracellular acidification depresses channel openings at a given Ca<sup>2+</sup><sub>i</sub> level, whereas an increase in Ca<sup>2+</sup> counteracts this acidification-induced reduction in the opening probability indicates that protons and Ca<sup>2+</sup> ions may compete for binding sites on the channel proteins (31). Indeed, acidification caused a decrease in the apparent Ca<sup>2+</sup> sensitivity. The gating mechanism of this channel is therefore controlled not only by Ca<sup>2+</sup><sub>i</sub> and membrane potential, but also by pH<sub>i</sub> in neocortical cells. It seems likely that H<sup>+</sup> binding, at least during hypoxia or ischemia, could decrease BK<sub>Ca</sub> channel activity despite an increase in Ca<sup>2+</sup><sub>i</sub>, considered adequate for activation of the channel.

*ATP, phosphorylation, and BK<sub>Ca</sub> channel activity.* Although it has been shown that ATP can modulate the activity of some BK<sub>Ca</sub> channel in rat brain (33), we did not find in the present study any significant effect of ATP on BK<sub>Ca</sub> channel when Ca<sup>2+</sup> concentration and pH in the perfusion system were tightly controlled. Thus, we exclude the possibility that ATP alone is involved in the hypoxia-induced inhibition of BK<sub>Ca</sub>. However, at least 3 type of kinases, including PKA, PKC, and cGMP-dependent protein kinase, have been reported to regulate BK<sub>Ca</sub>

channels through phosphorylation (13, 14, 32, 34, 44, 45). Using plasma membranes from rat brains, investigators have demonstrated the presence of at least 2 types of BK<sub>Ca</sub> channel: type I BK<sub>Ca</sub> channel, which is ChTX-sensitive, and type II BK<sub>Ca</sub> channel, which is insensitive to external application of ChTX. In addition, the activity of these 2 channels appears to be differentially regulated by protein kinases (13). Our channel in this work belongs to type II BK<sub>Ca</sub> channels because (a) it is resistant to ChTX and (b) channel activity is downregulated with a PKA catalytic subunit. This BK<sub>Ca</sub> channel may thus provide a link between different second messenger systems and the membrane properties of these neurons, particularly during hypoxia or ischemia. Data from the present study indicate that phosphorylation can alter the sensitivity of the channel to Ca<sup>2+</sup> and downregulate their open-state probability at physiological and relevant transmembrane voltage despite unchanged Ca<sup>2+</sup>. Furthermore, a correlation between phosphorylation and shift in the sensitivity to Ca<sup>2+</sup> of BK<sub>Ca</sub> channel may partly explain the relatively high variability of channel activation observed under identical conditions of membrane potential and cytosolic Ca<sup>2+</sup>. Because hypoxia is known to activate protein kinases (1), it is possible that these may in turn decrease Ca<sup>2+</sup> sensitivity and inhibit BK<sub>Ca</sub> channel via phosphorylation.

*Redox modulation and BK<sub>Ca</sub> channel activity.* It has been shown that redox regulation could influence K<sup>+</sup> channel activation (35, 46). This may be especially important during low O<sub>2</sub> or metabolic states as the redox potential of the cell is altered. Of interest, it has been shown in both carotid body cell and pulmonary arte-



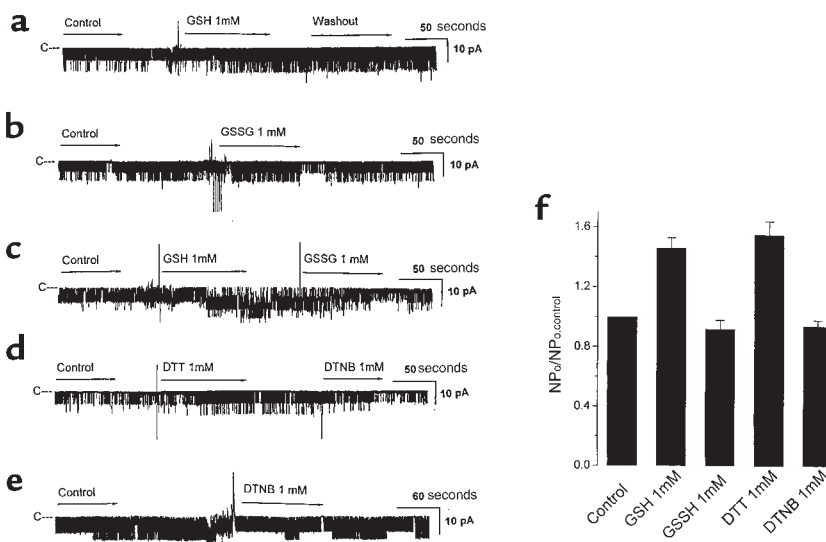
**Figure 9**

Effects of PKA and PKC activators on  $BK_{Ca}$  channel currents. Currents were recorded from a cell-attached patch of a neocortical neuron with high-KCl (140 mM) solution in the pipette and bathing outside solution, at a  $V_m$  of  $-20$  mV. (a) Application of 0.1 mM db cAMP markedly decreased the single  $BK_{Ca}$  channel activity and recovered after washout. (b) Application of 100 nM PMA had no apparent effect on  $BK_{Ca}$  channel activity. (c) Mean change in  $NP_o$ , measured in different conditions.

rial myocytes that reductants such as GSH or DTT can mimic the effect of hypoxia on  $O_2$ -regulated  $K^+$  channels (47, 48). Our results also demonstrated that  $BK_{Ca}$  channel in mice neocortical neurons can be modulated by changes in the redox environment. The reducing agents GSH and DTT enhance and stabilize the activity of  $BK_{Ca}$  channels, but no direct effect of oxidizing agents GSSG and DTNB on  $BK_{Ca}$  channel was observed in the present study. One possibility is that these channels were already in a fully oxidized state after excision of the patches. The channel's cysteines are usually exposed to the relatively reduced state in the cell interior because of ample GSH in the cytosol. However, after patch excision, some of these residues may be rapidly oxidized. Ion channels respond to redox reagents in diverse ways. For example, an oxidative environment causes the closure of ATP-sensitive  $K^+$  channels, and *hsl*o  $K_{Ca}$  channels, but it opens  $Ca^{2+}$  channels and removes inactivation in Kv1.4 channels (49). This diversity of redox effects

may be attributable to the reactivity of sulfhydryl groups of cysteine residues. The intracellular concentration of GSH range from 0.1 to 10 mM, and in the present study, 1 mM GSH augmented the  $BK_{Ca}$  channel activity significantly, suggesting that redox modification by GSH is likely to be of physiological relevance. The question is raised then as to whether this agent also participates in the hypoxia-induced inhibition on  $BK_{Ca}$  channel activity. We postulate that it will probably depend on how the intracellular GSSG/GSH ratio is altered during hypoxia.

*Other possible mechanisms.* Another potentially important factor is stress-activated proteolytic enzymes that may be involved in modulation of  $BK_{Ca}$  channel during hypoxia. The effects of trypsin and proteases were also explored in this regard. However, application of trypsin and proteases to the intracellular side of patches was not found to affect  $BK_{Ca}$  channel activity in the present experiments (data were not shown). Thus, we doubt the involvement of this process in  $BK_{Ca}$  channel activation.



**Figure 10**

Effect of the redox agents on the  $BK_{Ca}$  channel. Currents were recorded from inside-out patches of neocortical neurons, using symmetrical 140 mM KCl on both sides of the membrane,  $V_m$  of  $-30$  mV. (a) Application of 1 mM GSH markedly increased the single  $BK_{Ca}$  channel activity, and this activity persisted after washout. (b) Application of 1 mM GSSG had no apparent effect on  $BK_{Ca}$  channel activity. (c) The increase in channel activity caused by GSH (1 mM) recovered to control level when GSSG was applied. (d) Reducing agent DTT (1 mM) markedly augmented  $BK_{Ca}$  channel activity, which was reversed by the oxidizing agent DTNB (1 mM). (e) DTNB (1 mM) had no significant effect on the  $BK_{Ca}$  channel. (f) Mean change in  $NP_o$ , measured at different conditions.

Receptor modulation has also been important in hypoxia- or ischemia-induced release of endogenous transmitters or neurohormones, which might couple to receptors and modulate the channel activity. One substance known to be released in significant amount from ischemic brain is adenosine, and there is ample evidence that adenosine could regulate several types of K<sup>+</sup> channels by activation of its receptors (50, 51). However, it is not clear whether adenosine modulates BK<sub>Ca</sub> channel activity, and further studies are needed in this area.

*Functional role of BK<sub>Ca</sub> channel during hypoxia.* Two important questions remain unanswered concerning the BK<sub>Ca</sub> channel of mice neocortical neurons in relation to hypoxia: (a) what functional role(s) these channels have under physiological conditions, and (b) what the functional implications are of the hypoxia-induced inhibition of the BK<sub>Ca</sub> channels. These questions would seem to be crucial, because the frequency with which the BK<sub>Ca</sub> channel was observed in the mice neocortex was high and because its conductance is large. In central neurons, BK<sub>Ca</sub> channels have been implicated in the processes of spike repolarization and fast hyperpolarization after an action potential (52). These processes can clearly have a major effect on neuronal activity, frequency of firing, and neurotransmitter release, to name a few important aspects of neuronal function. Furthermore, in present study, this BK<sub>Ca</sub> channel was found to have high variability in its activity. The mechanisms for the high variability of BK<sub>Ca</sub> channel may be related to different phosphorylation level, Ca<sup>2+</sup> sensitivity, or a number of other factors discussed here. We believe that some of the main pathway for the regulation of BK<sub>Ca</sub> channel in mice neocortical neurons is related to a second messenger system other than Ca<sup>2+</sup>, e.g., cAMP-dependent phosphorylation of the channel protein. Hence, the activation of the BK<sub>Ca</sub> channel may require the combined effects of several factors, such as increased depolarization, intracellular alkalization, and decreased phosphorylation.

Depolarization in neocortical neurons appears usually a few to several minutes after anoxic exposure (1, 2), which is consistent with our findings of BK<sub>Ca</sub> channels inhibition. So the hypoxia-induced inhibition of BK<sub>Ca</sub> channel in central neurons, like in other tissues, may be involved in triggering membrane depolarization or in maintaining or accentuating it during hypoxia or ischemia. The depolarization during hypoxia leads to an elevation of intracellular free Ca<sup>2+</sup> which, in turn, can be potentially important for a number of cellular functions, including neurotransmitter release and activation of second messenger pathways. On the other hand, Ca<sup>2+</sup> overload can trigger a series of autotoxic cellular reactions and lead to the development of a vicious cycle with ultimate cell death. Therefore, it seems likely that O<sub>2</sub> modulation of ion channels is not only involved in cellular adaptive responses to hypoxia, but it also may participate in the pathophysiology of abnormal states. Indeed, the BK<sub>Ca</sub> channel we studied is so prevalent and

has such a high conductance that it may be considered a target for pharmacological interventions for treatment of hypoxic/ischemic diseases in the brain.

In summary, we have observed in the present study a hypoxia-induced inhibition of BK<sub>Ca</sub> channel activity in mice neocortical neurons. The mechanisms of this hypoxia-induced inhibition of BK<sub>Ca</sub> channel may be complex. However, we believe that the potential mechanisms mediating this reduction in activity during hypoxia are a reduced Ca<sup>2+</sup> sensitivity of the channel by cytosolic factors such as pHi and phosphorylation.

## Acknowledgments

The work is supported by National Institutes of Health grants HD 32573 and NS 35918.

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