ABSTRACT  The cascade of cellular events that is triggered by low O₂ levels in the central nervous system depends on initial sensing mechanisms that can be crucial in determining the overall cell response, adaptation, or injury. In this report, we demonstrate that the activity of an identified K⁺ channel is regulated directly by environmental O₂. Membrane ionic currents were recorded from neurons of the neocortex and the substantia nigra and studied by using whole-cell or excised membrane patches. O₂ deprivation reversibly induced an initial transient increase in whole-cell outward currents, and this was followed by a pronounced decrease in these currents. In cell-free excised membrane patches, lack of O₂ reversibly inhibited a class of K⁺ channels that are inhibited by ATP and activated by Ca²⁺. K⁺ channel inhibition depended on pO₂ level, with a 50% inhibition at ~11 torr (1 torr = 6.9 kPa). By the use of specific agents that chelate metal in metal-containing O₂-sensing centers, including heme, nonheme iron, copper, and flavin, we also demonstrated that iron-center but not copper-center blockers inhibited the channel in excised patches in a similar fashion as low pO₂. These results strongly suggest that K⁺ channel activity is modulated during O₂ deprivation by nonheme iron-containing proteins that are associated with channel molecules, thus providing evidence for a direct O₂-sensing mechanism in neuronal membranes.

It is widely accepted that cells, including neurons, sense low microenvironmental O₂ levels through alterations in the concentrations of cytosolic factors such as ATP or Ca²⁺. In central neurons, such alterations may have a major impact on membrane excitability by regulating K⁺ channel activity (1). However, recent findings from our laboratory have suggested that processes other than those originating in the cytosol of cells may underlie ion channel regulation during O₂ deprivation (2, 3). In central neurons, for example, we have found that hypoxia induces an initial increase in the whole-cell outward current but markedly suppresses it subsequently (2, 3). To test whether the effect of O₂ lack on K⁺ channel inhibition is mediated via mechanisms that are cytosol-independent and membrane-delimited, we performed the experiments described here.

MATERIALS AND METHODS

Rats (10–20 days) were deeply anesthetized with methoxyflurane and decapitated. The midbrain and cortex were rapidly removed, chilled in 0–1°C Ringer’s solution, and prepared as a tissue block. The tissue block was sectioned into 300-μm slices and incubated for 1 h with oxygenated Hepes buffer containing 140 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 25 mM D-glucose, 10 mM Hepes, and 0.2–0.3% trypsin (Sigma type XI) at 35°C (pH 7.35). Sections were then washed in oxygenated Hepes buffer and maintained for up to 6 hr. Immediately before recording, an individual tissue slice was removed and placed in a Na⁺-free Hepes buffer. Under a dissecting microscope, the substantia nigra nuclei or layers 4–5 of the temporal cortex were cut free and then dissociated by gentle and careful trituration with fire-polished Pasteur pipettes. Cells were plated in 35-mm Petri dishes and observed with Hoffman modulation optics. Recordings were only obtained from cells that did not show visible evidence of injury as illustrated in our previous publication (2).

Whole-cell outward currents were studied in the voltage-clamp mode (4) using patch pipettes (2–4 MΩ) and an Axon-patch C2 amplifier. Linear leak subtraction was performed on whole-cell current recordings. A Na⁺-free solution containing 150 mM choline chloride 2.5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 25 mM D-glucose, and 10 mM Hepes (pH = 7.4) was used in the bath; the internal solution contained 150 mM KCl, 0.2 mM MgCl₂, 25 mM D-glucose, and 10 mM Hepes (pH = 7.4). Single-channel currents were recorded from inside-out patches (4). Current records were low-pass filtered (Bessel, 4-pole filter, ~3 decibels at 2 kHz), digitized (10 kHz, 12-bit resolution) using an Axon-patch C2 amplifier, and stored on computer disk for later analysis. The same solutions were used in the bath and in recording pipettes, and these contained 150 mM KCl, 0.2 mM MgCl₂, 0.98 mM CaCl₂, 25 mM D-glucose, 10 mM Hepes, and 1 mM EGTA (pH = 7.4, calculated Ca²⁺ = 2 μM). Single-channel data were processed (0–750 Hz) with a Gaussian filter using PCLAMP 5.5.1 (Axon Instruments, Burlington, CA). The current amplitude was fitted with Gaussian distributions, and the difference between two adjacent fitted peaks was taken as unitary current amplitude (5, 6).

Graded hypoxia was induced by perfusing patches with one of the solutions bubbled for at least 2 hr with 2%, 1%, 0% O₂ (balanced with N₂), or 100% N₂ plus 2 mM Na₂S₂O₄. pO₂ levels in these solutions were measured with polarographic electrodes (8 μm in diameter) (7) and averaged 14.1 ± 0.6 (n = 5), 7.6 ± 0.3 (n = 8), and 0 ± 0.1 (n = 5) torr, respectively. Na₂S₂O₄ was used only in experiments in which a pO₂ < 1 torr was needed.

RESULTS AND DISCUSSION

Whole-cell outward currents were recorded from neocortical and substantia nigra neurons in voltage-clamp mode. Substantia nigra and neocortical neurons were chosen because (i) a number of K⁺ channels have been demonstrated in these nerve cells by us and others (3, 8, 9); and (ii) there is a high density of glibenclamide-binding sites in these two brain areas (10, 11). When these neurons were exposed to an anoxic medium (measured pO₂ < 1 torr), outward current amplitude showed an initial transient increase (2, 3), which was followed by a decrease that reached 32 ± 3.9% (mean ± SEM, n = 8 cells) below baseline level (Fig. 1). After washout with a perfusion medium of pO₂ ~150 torr, current amplitude...
FIG. 1. Whole-cell outward currents were reversibly inhibited during anoxia. (A) The cell was held at -70 mV and stepped to 40 mV every 5 sec (lower trace). Recordings during baseline (measured pO₂ = 150 torr), anoxia (pO₂ < 1 torr), and reperfusion (pO₂ = 150 torr) are superimposed; each trace is an average of 16 recordings. (B) Percentage decrease in current amplitude. About 30% decrease in outward currents was observed (n = 8 cells), and the change was statistically significant (*: P < 0.001, paired Student t test). Data are presented as mean ± SEM.

returned to baseline, demonstrating that this decrease in outward current was reversible.

To study the ionic basis of this O$_2$-sensitive current and determine whether membrane-delimited mechanisms participate on O$_2$ sensing, single-channel currents were recorded from inside-out patches of acutely dissociated neurons. We have characterized one of the K$^+$ currents using ion substitution and reversal potentials (3). This channel has high selectivity to K$^+$ and is voltage-activated (membrane potential for 50% maximum activation is 13 mV) and Ca$^{2+}$-dependent (IC$_{50}$ = 14 μM). The channel has a large conductance (~200 pA) and is inhibited by ATP (IC$_{50}$ = 135 μM) and 5'-adenylylimidodiphosphate, a nonhydrolyzable ATP analogue. When inside-out patches containing this ATP-sensitive K$^+$ channel were exposed to an internal medium with an average pO$_2$ of ~8 torr, the channel open-state probability ($P_{open}$) started to decrease within 1 min. A maximum inhibition was reached in 2–3 min when $P_{open}$ was reduced to a mean of ~25% of control values (Fig. 2A). Unitary conductance was also decreased but generally not to the same degree as $P_{open}$ (~80% of control). In addition, a characteristic increase in channel open–closing frequency and rapid channel flickerings was observed (Fig. 2A). After reperfusion with a medium of pO$_2$ = 150 torr, >90% of the patches showed a recovery of $P_{open}$. This hypoxia-induced inhibition of channel activity depended on the pO$_2$ level. Channel activity was almost totally inhibited when pO$_2$ was <1 torr, but the channel was unaffected when pO$_2$ ≥ 20 torr. The pO$_2$ level corresponding to 50% inhibition of channel $P_{open}$ was ~11 torr (Fig. 2B).

In sharp contrast, hypoxia (~8 torr) did not alter $P_{open}$ or unitary conductance of the Ca$^{2+}$-independent, smaller conductance (40 pS) K$^+$ channel (Fig. 3) that is often seen in these cells (n = 3). Even lower pO$_2$ (<1 torr) was ineffective (n = 2), suggesting that neuronal K$^+$ channels were not all sensitive to lack of O$_2$.

Because low pO$_2$ inhibited the ATP-sensitive, large-conductance K$^+$ channel in the absence of cytosolic components, we argued that membrane-delimited mechanisms were involved in this O$_2$-mediated regulation of K$^+$ channel activity. In an attempt to elucidate such mechanisms, we used agents that alter the redox state of membrane proteins in cell-free patches. We found that reduced glutathione inhibited the same K$^+$ current in substantially the same way as hypoxia because reduced glutathione inhibited $P_{open}$ and increased channel open–closing frequency and flickering activity (n = 5) in both inside–out and outside–out configurations. Oxidized glutathione, in concentrations of 0.5 and 1.0 mM, had no effect. Another redox agent, reduced dihydrothreitol (5 mM), also significantly inhibited $P_{open}$ of this K$^+$ current (n = 3).

There are at least two potential models that can explain the redox-related O$_2$ sensitivity of this K$^+$ channel: (i) a decrease in pO$_2$ levels depresses the concentration of oxidant species (e.g., H$_2$O$_2$), which, in turn, can attenuate K$^+$ channel activity (12–14) and (ii) O$_2$ deprivation induces a change in the channel molecular conformation via a change in the redox state of channel molecules (15, 16) or via interactions with a metal-containing center such as iron–containing proteins (17). To address the first hypothesis, we applied hemoglobin (18)
**Fig. 3.** Oxygen deprivation does not affect channel activity of a small-conductance K⁺ current. Continuous recordings of a single K⁺ current from an inside-out patch using an equal concentration of K⁺ (150 mM) on both sides of the membrane with membrane potential held at 40 mV. Straight lines indicate channel closure. During baseline (top trace), this channel had a $P_{open}$ of 0.81 (from a 18-sec stretch of data). Hypoxia does not induce an inhibition of channel activity ($P_{open} = 0.89$, middle trace). After reperfusion (with a normoxic medium (P₂ = 150 torr), $P_{open}$ remains at the same level as during hypoxia (0.89, lower trace). Single-channel conductance (38 pS) is maintained at the same level before, during, and after hypoxia.

(250 μg/ml, n = 5) and N-acetylcysteine (19) (1–2 mM, n = 5) to the internal surface of inside-out patches. We found that these agents had no effect on K⁺ channel activity (both $P_{open}$ and conductance) (Fig. 4B). Using diphenyliodonium (10 μM), a specific blocker of NADH oxidase (flavoprotein) (20) that catalyzes the production of H₂O₂, we observed neither a significant change in K⁺ channel $P_{open}$ nor in conductance (Fig. 4B). In addition, because N-acetylcysteine and hemoglobin have a high-affinity carbon monoxide (CO), these experiments suggest that CO does not appear to be involved in the O₂-sensing phenomenon.

To determine whether metal-containing proteins are involved in this O₂-sensing mechanism, we used several metal-center blockers (or chelators) in inside-out patches, keeping pO₂ ~150 torr. Heme, nonheme iron, copper, and flavin were chosen, as these O₂-binding metal centers represent >90% of all known centers that interact with O₂. We found that the copper-center blocker bathocuproinedisulfonic acid (100 μM) (21, 22) had no effect on either K⁺ channel $P_{open}$ or conductance. However, 1,10-phenanthroline (1 mM), a preferential iron-center blocker (23, 24), strongly inhibited $P_{open}$ (Fig. 4A) in a similar fashion as O₂ deprivation or reduced glutathione in both inside-out and outside-out patches. This inhibitory effect is not mediated through chelating Ca²⁺ because we found that the inhibitory effect of 1,10-phenanthroline (1 mM) was not influenced by the presence of a high concentration of Ca²⁺ (2 mM, n = 3). In addition, deferoxamine (2 mM), another iron-center blocker (25) and potassium cyanide (0.5–2.0 mM), known to bind to iron-containing proteins, also suppressed $P_{open}$ significantly (Fig. 4A). Interestingly, a medium containing CO (pCO = 380 torr) did not have any effect on channel $P_{open}$ or conductance. Therefore, these results strongly suggest that K⁺ channel activity in neuronal membranes can be regulated by microenvironmental O₂ levels through metal-containing proteins. In sharp contrast, the smaller conductance K⁺ channel was not affected by these metal chelators. We did not see any inhibition of $P_{open}$ or conductance when patches containing the smaller K⁺ currents were exposed to 1,10-phenanthroline (1 mM, n = 4).

That neuronal plasma membranes have an O₂-sensing mechanism that is mediated by a metal-binding protein is not only a surprising finding but also constitutes, we believe, an unusual concept. This is particularly significant for neurons because the mode of O₂ sensing that we observed in this study is via K⁺ ion channels that can affect neuronal excitability in a major way. Because our results point to the uniqueness of certain K⁺ channels, we argue that there is a specific interaction(s) between these O₂-sensitive K⁺ channels and metal-binding proteins in neuronal plasma membranes.

Although it is possible that glomus cells, the main cells in the chemosensory carotid body, transduce O₂ by membrane mechanisms (26, 27), there are at least two major differences between our results on neuronal membranes and those on glomus cells. (i) Glomus cells seem to be sensitive to a pO₂ that is much higher than that for nerve cells (26, 27). Indeed, the change in single-channel $P_{open}$ of glomus cells occurs between 150 and 70 torr (26, 27), whereas the neuronal K⁺ channel is sensitive to a pO₂ of <20 torr. Because interstitial pO₂ in the brain is generally ~25–50 torr, our findings become physiologically significant since this mechanism of O₂ sensing operates just below this pO₂ range during episodes of O₂.

**Fig. 4.** (A) Channel activity is reversibly inhibited by 1,10-phenanthroline. Currents were recorded in the same condition as Fig. 2. During baseline (top two traces), this K⁺ channel had a $P_{open}$ of 0.92. $P_{open}$ was reduced to 0.48 when the internal side of the patch was exposed to 1 mM 1,10-phenanthroline (middle two traces). This value was followed by a recovery ($P_{open}$ = 0.89) after washout (lower two traces). (B) Effects of different agents on $P_{open}$. GSH, reduced glutathione (n = 6); DTT, reduced diithiothreitol (n = 5); PTL, 1,10-phenanthroline (n = 7); CN, potassium cyanide (n = 4); BCS, bathocuproinedisulfonic acid (n = 6); HGB, hemoglobin (n = 5); DPI, diphenyliodonium (n = 7), CO, carbon monoxide (n = 4). Stars indicate that changes are significantly different from baseline levels ($P < 0.05$).
deprivation. (ii) There is increased evidence that NAD(P)H oxidase, which is present in peripheral chemoreceptor cell membranes, is a favorite candidate for an O₂ sensor (12, 14). Therefore, it is believed that an NAD(P)H oxidase (flavoprotein) that catalyzes the production of H₂O₂ plays a central role in O₂-sensing mechanisms in glomus cells (20). In the present study, we did not find any change in channel activity after the inhibition of NAD(P)H oxidase, suggesting that the H₂O₂-generating system by NAD(P)H oxidase does not seem to be involved in channel regulation in neurons. In addition to H₂O₂, other reactive oxygen species such as superoxides do not seem to be involved because our data indicate that high-affinity reactive oxidant scavengers (acetylcysteine and hemoglobin) do not affect channel activity. Our data strongly suggest that O₂ sensing is conducted by a different process that involves a metal-binding protein. This metal-containing protein is likely to be a nonheme protein that has a high affinity to iron chelators—e.g., phenanthroline, cyanide, and deferoxamine but not to CO.

Although whole-cell recordings show that low pO₂ induces an initial increase in the same K⁺ current, we believe that the membrane-delimited mechanisms that are activated in this early period dampen the increase in this K⁺ current. Because this channel is strongly activated when intracellular ATP decreases and Ca²⁺ increases (3), the predominant effect of O₂ deprivation on channel activity in the initial stage of hypoxia would seem to enhance this current. Most likely, the inhibitory action starts to be manifested only when the maximum effect of the membrane-delimited mechanisms occur.

This O₂-sensing mechanism, which is based on K⁺ channel inhibition, can have a major impact on neuronal excitability. Because the direct effect of O₂ lack on neuronal membranes is depolarizing in nature, this mechanism can counteract some of the effects induced by cytosolic alterations resulting from hypoxia, which are generally inhibitory to neuronal activity (3). For example, the decrease in ATP during metabolic stress can activate the same O₂-sensitive K⁺ channels (3), tending to hyperpolarize neurons. This dichotomy between excitation and inhibition could endow nerve cells with an exquisite ability to modulate their membrane excitability as a function of severity and duration of O₂ deprivation and as a function of the temporal diffusion of molecular O₂ in their microenvironment.

We thank Drs. Joseph Coleman, Fred Sigworth, and Joseph Warshaw for helpful discussions of this work. We also thank Dr. Sergio Grunstein at the University of Toronto for providing diphenyliodonium. This work was supported by National Institutes of Health Grants HL 39924, HD 28894, and HD15736. C.J. is a fellow of the Parker Francis Family Foundation.