# Oxygen deprivation inhibits a $K^+$ channel independently of cytosolic factors in rat central neurons

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1. $K^+$ channel modulation has been shown to be an integral and important cellular response to $O_2$ deprivation. Although part of this modulation occurs as a result of changes in concentrations of several cytosolic factors such as ATP and $Ca^{2+}$, it is unknown whether there are mechanisms other than those originating from the cytosol. To test the hypothesis that membrane-delimited mechanisms participate in the $O_2$-sensing process and are involved in the modulation of $K^+$ channel activity in central neurons, we performed experiments using patch-clamp techniques and dissociated cells from the rat neocortex and substantia nigra.

2. Whole-cell outward currents were studied in voltage-clamp mode using $Na^+$-free or low-$Na^+$ (5 mM, with 1 $\mu$M tetrodotoxin) extracellular medium plus 0·5 mM $Co^{2+}$. $O_2$ deprivation produced a biphasic response in current amplitude, i.e. an initial transient increase followed by a pronounced decrease in outward currents. The reduction in outward currents was a reversible process since perfusion with a medium of $P_{O_2} > 100$ mmHg (1 mmHg = 133 Pa) led to a complete recovery.

3. In cell-free excised membrane patches, we found that a specific $K^+$ current (large conductance, inhibited by micromolar concentrations of ATP and activated by $Ca^{2+}$) was reversibly inhibited by lack of $O_2$. This was characterized by a marked decrease in channel open-state probability and a slight reduction in unitary conductance. The magnitude of channel inhibition by $O_2$ deprivation was closely dependent on $O_2$ tension. The $P_{O_2}$ level for 50% channel inhibition was about 10 mmHg with little or no inhibition at $P_{O_2} \geqslant 20$ mmHg.

4. Single-channel kinetic analysis showed that channel open times consisted of two components and closed times were composed of three. The hypoxia-induced inhibition of $K^+$ channel activity was mediated by selective suppression of the longer time constant channel openings without significantly affecting closed time constants. This led to an increase in frequency of opening and closing and rapid channel flickerings.

5. Our data showed that $O_2$ deprivation had no effect on another $K^+$ current characterized by a much smaller conductance and $Ca^{2+}$ independence. This provides evidence for the selective nature of the hypoxia-induced inhibition of some species of $K^+$ channels.

6. These results therefore provide the first evidence for regulation of $K^+$ channel activity by $O_2$ deprivation in cell-free excised patches from central neurons.

It has recently been recognized that the consequences of a hypoxic or ischaemic episode are not only determined by the severity and duration of $O_2$ and nutrient deprivation but are also influenced by the cellular and membrane functions of cells (Hochachka, 1986; Leblond & Krnjević, 1989; Gonzalez, Lowenstein, Fernyak, Hisanaga, Simon & Sharp, 1991; Rordorf, Koroshetz & Ronventre, 1991; Jiang & Haddad, 1991; Murphy & Greenfield, 1992; Xia, Jiang & Haddad, 1992; Cummins, Jiang & Haddad, 1993; Haddad & Jiang, 1993*b*; Kaku, Giffard & Choi, 1993). One of the hypoxia-induced functional alterations in mammalian central neurons, cells that are extremely vulnerable to $O_2$ deprivation, is the modulation of membrane $K^+$ channel activity. Since $K^+$ channels are known to play a key role in the maintenance of membrane potential and in neuronal excitability (Hille, 1992), the regulation of $K^+$ channel activity is believed to have a major impact on the overall neuronal response and adaptation to $O_2$ deprivation (Haddad & Jiang, 1993*a*). In some neurons, for example, activation of $K^+$ channels

during hypoxia induces hyperpolarization, decrease in membrane excitability and reduction in $O_2$ consumption (Krnjević & Leblond, 1989; Leblond & Krnjević, 1989; Murphy & Greenfield, 1992). On the other hand, a prolonged period of activation of $K^+$ channels can produce an accumulation of $K^+$ in the interstitial fluid, which will thus increase the ratio of $[K^+]_o/[K^+]_i$. According to the Nernst equation, this will lead to depolarization and an increase in membrane excitability, thus accelerating energy consumption (Hansen, 1985; Jiang & Haddad, 1991; Jiang & Haddad, 1992; Jiang, Xia & Haddad, 1992).

It has been well documented that the regulation of $K^+$ channel activity during hypoxia is mediated via changes in concentrations of cytosolic factors such as ATP and $Ca^{2+}$ (Haddad & Jiang, 1993*a*). Recent studies, however, have shown that hypoxia induces an inhibition of $K^+$ currents in some excitable cells such as carotid glomus cells, pulmonary arterial myocytes and pulmonary neuroepithelial body cells (Ganfornina & López-Barneo, 1991, 1992; Post, Hume, Archer & Weir, 1992; Youngson, Nurse, Yeger & Cutz, 1993; Yuan, Goldman, Tod, Rubin & Blaustein, 1993), and this may be mediated by mechanisms other than those originating from the cytosol (Ganfornina & López-Barneo, 1991, 1992). Also, our previous studies have suggested that such $O_2$-sensing mechanisms may also be present in central neurons (Jiang & Haddad, 1993). For example, we have found that $O_2$ deprivation induces an initial increase in $K^+$ current which is likely to be mediated by a decrease in intracellular ATP level and an increase in cytosolic free $Ca^{2+}$ concentration (Jiang, Sigworth & Haddad, 1994). In addition, when hypoxia is prolonged, there is a marked decrease in whole-cell $K^+$ current following the initial increase (Jiang & Haddad, 1993). The reason for this $K^+$ current attenuation is not understood but it is possible that it is mediated by factor(s) other than ATP and $Ca^{2+}$ (Jiang & Haddad, 1993; Jiang *et al.* 1994).

Other potential mechanisms for this hypoxia-induced reduction in $K^+$ current could include a direct effect of $O_2$ deficiency on neuronal $K^+$ channels via cytosol-independent and membrane-delimited processes. To test this hypothesis, we designed experiments using cell-free excised membrane patches and studied the effect of $O_2$ deprivation on specific $K^+$ currents previously characterized in the substantia nigra and in the neocortex (Jiang *et al.* 1994). Our results clearly showed that the activity of this $K^+$ channel, which has a large conductance and is inhibited by ATP, was greatly reduced during $O_2$ deprivation in cell-free excised patches obtained from central neurons.

## METHODS

Neurons were harvested from the substantia nigra zona compacta (SN) of Sprague–Dawley rats (10–20 days old) using the modified methods of Kay & Wong (1986). In some experiments, neurons were also obtained from the rat temporal cortex. Since responses to hypoxia of these cortical neurons were similar to those of SN cells and since data from cortical neurons formed a rather small portion of the total results, we did not separate these neurons into an independent group. In brief, rats were deeply anaesthetized with methoxyfluorane (saturated) and decapitated. The midbrain was rapidly removed, chilled in Ringer solution (Jiang & Haddad, 1991) at 0–1 °C and prepared as a tissue block. The tissue block was sectioned transversely into 250 $\mu$m slices. Sections were incubated for 1 h with oxygenated Hepes buffer containing (mM): NaCl, 140; KCl, 2·5; $MgCl_2$, 1; $CaCl_2$, 1; D-glucose, 25; Hepes, 10 and trypsin (0·2–0·3%, Sigma Type XI, Sigma, St Louis, MO, USA) at 35 °C (pH 7·36). Sections were then washed in oxygenated Hepes buffer and maintained for up to 6 h.

Immediately prior to recording, individual tissue slices were removed and placed in a $Na^+$-free Hepes buffer. Using a dissecting microscope, the substantia nigra pars compacta on both sides and layers 3–5 of neocortex were cut free from the rest of the slice. The tissue of interest was then dissociated by gentle and careful trituration with fire-polished Pasteur pipettes. Cells were plated in 35 mm Petri dishes and observed with Hoffman diffraction optics. Recordings were only obtained on cells which exhibited the morphology of catecholaminergic cells in the substantia nigra as has been described previously (Domesick, Stinus & Paskevich, 1983; Grace & Onn, 1989; Murphy & Greenfield, 1992; Jiang *et al.* 1994) and pyramidal cells from the neocortex. Flat or swollen cells or cells with a grainy membrane appearance were considered as injured and were not studied (Kay & Wong, 1986; Cummins, Jiang & Haddad, 1993).

Patch-clamp experiments were performed at room temperature (about 24 °C). Fire-polished patch pipettes (2–4 MΩ) were made from 1·2 mm borosilicate capillary glass (Sutter P–80/PC puller, Sutter Instruments Co., Novato, CA, USA). Single-channel currents were recorded from inside-out or outside-out patches. Whole-cell outward currents were studied in the voltage-clamp mode using the whole-cell configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Current records were low-pass filtered (0–2 kHz, Bessel 4-pole filter, −3 dB), digitized (10 kHz, 12-bit resolution) using an Axo-patch C2 amplifier, and stored on computer disk for later analysis (pCLAMP 5.5.1, Axon Instruments Inc., Foster City, CA, USA). Linear leak subtraction was performed on whole-cell current recordings.

For single-channel recordings, the same solutions were applied to the bath and recording pipettes, and these contained (mM): KCl, 150; $MgCl_2$, 0·2; $CaCl_2$, 0·98; D-glucose, 25; Hepes, 10 and EGTA, 1 (pH 7·4, free $Ca^{2+}$ 2 $\mu$M). For whole-cell recordings, a $Na^+$-free solution containing (mM): choline chloride, 150; KCl, 2·5; $MgCl_2$, 1; $CaCl_2$, 1; D-glucose, 25 and Hepes, 10 (pH = 7·4) was used in the bath with the same pipette solution as for single-channel recordings. In several control experiments, *N*-methyl-D-glutamine (NMDG) was also used as a $Na^+$ substitute, and we did not see any difference in channel activity and conductance when choline *versus* NMDG was used. A perfusion system was used to administer agents to the recorded patches or cells with a speed of ~1 ml $min^{-1}$. Graded hypoxia was instituted by perfusing with solutions bubbled for at least 2 h with 2, 1 or 0% $O_2$ (balanced with $N_2$). Anoxia was produced by a solution bubbled with 100% $N_2$ in addition to $Na_2S_2O_4$ (2 mM), which acts as an $O_2$ sink. The $P_{O_2}$ levels in these four solutions were measured with polarographic electrodes (8 $\mu$m in diameter; Jiang, Agulian & Haddad, 1991), and averaged 18·7 ± 0·8

($n = 5$), 14·1 ± 0·6 ($n = 5$), 7·6 ± 0·3 ($n = 8$) and 0 ± 0·1 ($n = 5$) mmHg (1 mmHg = 133 Pa), respectively. Stable $P_{O_2}$ levels were reached in 15–20 s. Since these $P_{O_2}$ levels were consistently obtained from different experiments with different electrodes, we used these values as the $P_{O_2}$ levels reached. $Na_2S_2O_4$ was added only in experiments with $P_{O_2} < 1$ mmHg.

For single-channel analysis, data were further filtered (0–750 Hz) with a Gaussian filter. In order to ascertain the amplitude of current that showed a rapid flickering during hypoxia (see Results), data were also analysed without further filtering (i.e. 0–2 kHz). Openings shorter than 100 $\mu$s were not included. The open-state probability ($P_{open}$) was calculated by measuring the time, $t_j$, spent at current levels corresponding to $j = 0, 1, 2....N$ channels open (Quayle, Standen & Stanfield, 1988; Davies, Standen & Stanfield, 1992). The $P_{open}$ was obtained using

$$P_{open} = \left(\sum_{j=1}^{N} t_j j\right) \Big/ TN,$$

where $N$ is the number of channels active in the patch and $T$ is the duration of recordings. $P_{open}$ was calculated from data duration > 18 s. Open and closed times were measured from records in which only a single channel was active. The open- and closed-time distributions were fitted using the method of maximum likelihood (Colquhoun & Sigworth, 1983; Sigworth & Sine, 1987). The current amplitude was described using Gaussian distributions and the difference between two adjacent fitted peaks was taken as unitary current amplitude. Missed events (brief and substate closures) were very small (they were estimated by comparing dwell-time histograms with the fitted profile to be roughly < 0·003% of the total area of the open-time histogram and < 0·1% of that of the closed-time histogram).

Data are presented as means ± S.E.M. ($n$ = number of patches) and differences in means were tested with Student's $t$ test and $\chi^2$ and were accepted as significant if $P \leqslant 0.05$.

## RESULTS

### Inhibition of whole-cell outward current during hypoxia

Whole-cell outward current was studied in voltage-clamp mode, after elimination of inward $Na^+$ and $Ca^{2+}$ currents using a pipette solution containing 150 mM KCl and an external solution containing 0·5 mM $Co^{2+}$ and 0 mM $Na^+$ ($Na^+$ was substituted by $choline^+$) or 0·5 mM $Co^{2+}$ and 5 mM $Na^+$ with 1 $\mu$M tetrodotoxin. Cells were held at −70 mV and stepped to 40 mV with depolarizing pulses every 5 s. Under this condition, only outward currents

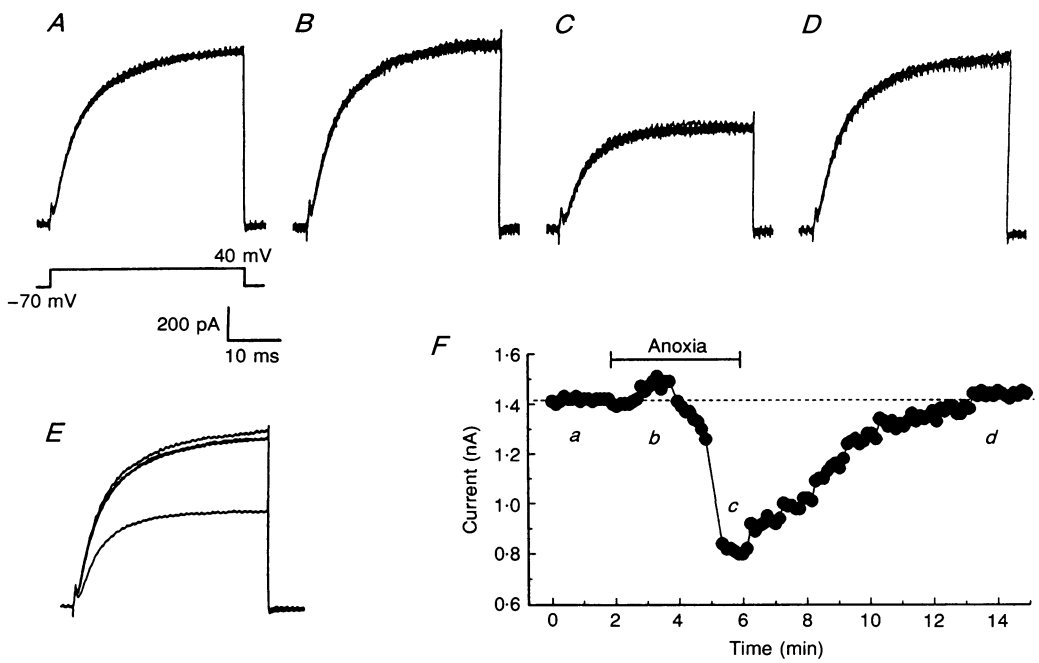


**Figure 1. Alterations in whole-cell outward currents of a SN neuron during hypoxia**
A solution containing 150 mM $K^+$ was used in the pipette and a $Na^+$-free (replaced by choline) medium was applied to cells. The cell was held at −70 mV and stepped to 40 mV every 5 s (lower trace in *A*). Superimposed are 8 traces shown in *A* (control), *B* (2 min into anoxia), *C* (4 min into anoxia) and *D* (6 min after reperfusion). *E*, to compare hypoxia-induced changes in outward current, averaged recordings in *A*, *B*, *C* and *D* are superimposed with the largest one from *B* and smallest one from *C*. *F*, time profile of the current amplitude of this cell before, during and after anoxia. Each point represents an average of the last 15 ms of a single trace. *A*, *B*, *C*, and *D* were taken from *a*, *b*, *c* and *d*, respectively. The dashed line indicates the baseline current level. Note the initial increase (*b*) and the major late decrease (*c*) in current amplitude. The start time of hypoxia was counted as the time when the hypoxic solution was switched on.

were recorded from these neurons (Fig. 1). Using a pipette solution containing 140 mM TEACl and 10 mM CsCl, we found that more than 90% of the voltage-dependent outward current was suppressed ($n = 4$), suggesting that the overwhelming majority of the outward current is carried by $K^+$.

When cells were exposed to low-$O_2$ medium (measured $P_{O_2} < 1$ mmHg), there was an initial increase (Fig. 1*B*) followed by a marked decrease in the amplitude of the outward current (Fig. 1*C*). The increase in outward current started about 1 min after the onset of hypoxia and lasted for 1–2 min, reaching a maximum of 10–15% ($P < 0{\cdot}01$, $n = 8$) over baseline level. When cells were deprived of $O_2$ for a more prolonged period (5–6 min), the outward current was markedly decreased by $32 \pm 3{\cdot}9$% (mean ± S.E.M., $P < 0{\cdot}001$, $n = 8$ cells; Fig. 1*E* and *F*). This hypoxia-induced depression in outward current was reversible, since current amplitude returned to baseline level after perfusing with a medium of $P_{O_2} \approx 150$ mmHg (Fig. 1*D* and *F*).

The initial increase in $K^+$ current during hypoxia is likely to be mediated by alterations in intracellular nucleotide concentrations or levels of other cytosolic components (e.g. $Ca^{2+}$ and $H^+$), as we have previously described (Jiang *et al.* 1994). In order to study (1) the mechanism(s) for the subsequent $K^+$ current inhibition and (2) the direct potential effects of $O_2$ (or lack thereof) on neuronal membranes, patches were excised and studied in the absence of the cytosol.

## Hypoxia-induced inhibition of a $K^+$ current in cell-free excised patches

Single-channel currents were recorded from inside-out patches of central neurons. We focused on two distinct outward currents in the present study. One of the currents had a large conductance (~200 pS) and the other a small conductance (~40 pS). Both of these outward currents were highly selective for $K^+$ rather than $Na^+$ (Figs 2 and 3) and showed a sustained activation pattern with stepped depolarizing pulses (Figs 2*C* and 3*C*). The $K^+$ channel with

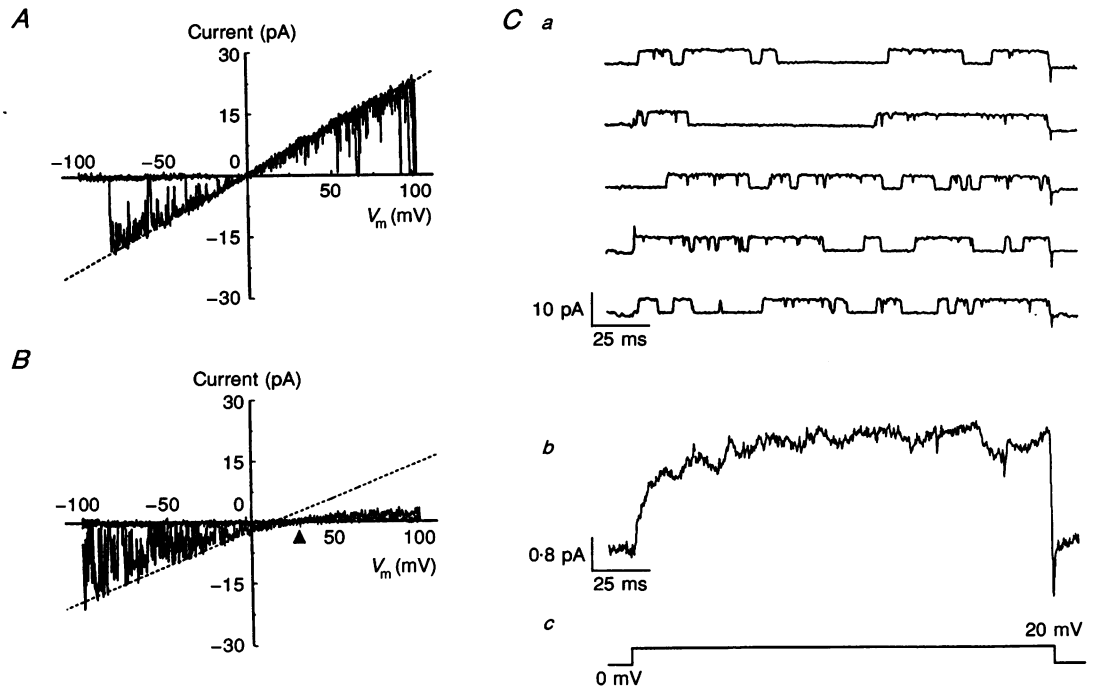


**Figure 2. The large-conductance current is carried by $K^+$ current**
*A*, current–voltage (*I–V*) relation of the large-conductance outward current from a SN neuron. Current was recorded with an equal concentration of $K^+$ (150 mM) on both sides of the membrane of an inside-out patch. *A*, this channel had a linear conductance of 229 pS (dashed line) in the voltage range −100 to 100 mV and a reversal potential of 0 mV. *B*, three superimposed traces. When bath $K^+$ concentration was reduced to 50 mM by substitution with 100 mM NaCl, the reversal potential of this current was shifted to approximately 25 mV (arrowhead) and showed inward rectification at positive potentials. At negative potentials from −100 to 0 mV, the unitary conductance of this channel was almost linear (169 pS, dashed line). *C*, reconstruction of the 'macroscopic' current. Current was recorded from an inside-out patch of another SN neuron with an equal concentration of $K^+$ in the bath and pipette. The patch was held at 0 mV and stepped to 20 mV (*c*). In this condition, a single active channel was seen (*a*). After averaging 288 individual traces, a sustained outward current was obtained (*b*).

the large conductance is voltage-dependent and its activation requires the presence of $Ca^{2+}$; this channel is also inhibited by physiological concentrations of ATP (Fig. 4). The small $K^+$ current was $Ca^{2+}$- and ATP-independent.

After excision, the large-conductance $K^+$ current appeared in about one third of inside-out patches. Identification of this current was routinely performed before further studies, and only those currents that had a large conductance (~200 pS) and were inhibited by ATP were used in these experiments. When patches were exposed to a low $O_2$ medium ($P_{O_2} = 0$–8 mmHg), single-channel activity of these currents was markedly inhibited. The inhibitory effect of low $P_{O_2}$ was characterized by a decrease in $P_{open}$ and an increase in channel open–close frequency resulting in rapid flickering (Fig. 5*A*). Channel inhibition started about 1 min after the onset of hypoxia and maximum inhibition was reached in about 3 min (Fig. 5*B*). With a $P_{O_2}$ level of ~8 mmHg, $P_{open}$ was markedly reduced to $25 \pm 5\%$ ($n = 19$) of baseline level. Reperfusion with oxygenated internal medium ($P_{O_2} \approx 150$ mmHg) led to partial or complete recovery in most patches (15/19). On average, $P_{open}$ recovered to $67 \pm 8\%$ ($n = 19$) of the baseline level 4–6 min after reoxygenation (Fig. 5*B*).

The inhibitory effect of hypoxia on $P_{open}$ was directly related to $P_{O_2}$ levels. Channel activity was almost totally suppressed with a $P_{O_2}$ level of < 1 mmHg, while the channel was unaffected when $P_{O_2} \geqslant 20$ mmHg. The $P_{O_2}$ level for 50% inhibition of $P_{open}$ was about 10 mmHg (Fig. 6).

Figure 7 shows the change in current amplitude during hypoxia. Two active channels were seen during baseline recording and both of them had a similar current amplitude (5·1 pA with the membrane potential ($V_m$) held at 25 mV). $O_2$ deprivation not only suppressed $P_{open}$ (from 0·98 to 0·24) but also significantly inhibited single-channel conductance. When the $P_{O_2}$ level was ~8 mmHg, the unitary conductance was reduced to an average of $69 \pm 6\%$ ($n = 17$) of its control level.

In sharp contrast, channel activity of the smaller conductance $K^+$ channel did not show any significant change ($P > 0{\cdot}05$, $n = 5$) during $O_2$ deprivation ($P_{O_2} \approx 8$ mmHg),

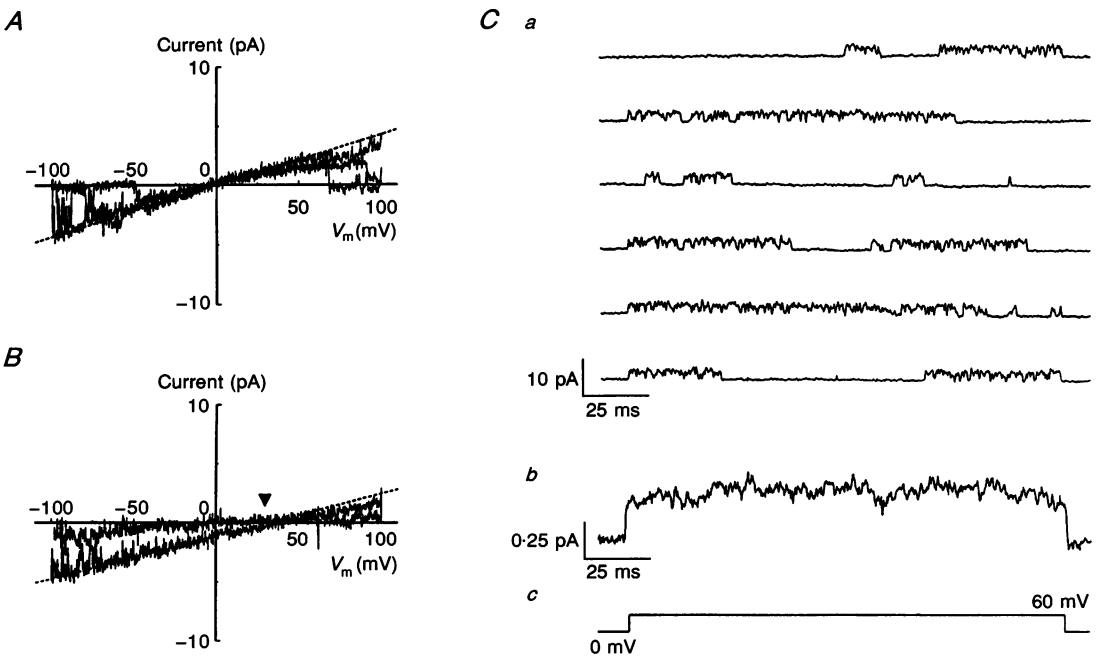


**Figure 3. The small-conductance current is also a $K^+$ current**
*A*, *I–V* relation of the small-conductance outward current from a SN neuron. Current was recorded with equal concentration of $K^+$ (150 mM) on both sides of the membrane of an inside-out patch. *A*, this channel had a linear conductance of 43 pS (dashed line) in the voltage range −100 to 0 mV and a reversal potential of 0 mV (three superimposed traces). *B*, when bath $K^+$ concentration was reduced to 50 mM by substitution with 100 mM NaCl, the reversal potential of this current was shifted to near 26 mV (arrowhead). The unitary conductance remained linear at negative potentials with a slope of 36 pS (dashed line). Note that there was another small current recorded in this patch, whose conductance was ~10 pS and which was not observed at a $V_m$ of 40 or 60 mV. *C*, reconstruction of the macroscopic current. Current was recorded from the same patch with equal concentration of $K^+$ in the bath and pipette. When the patch was held at 0 mV and stepped to 60 mV (*c*), a single active channel was seen (*a*). After averaging 300 individual traces, a sustained outward current was obtained (*b*).

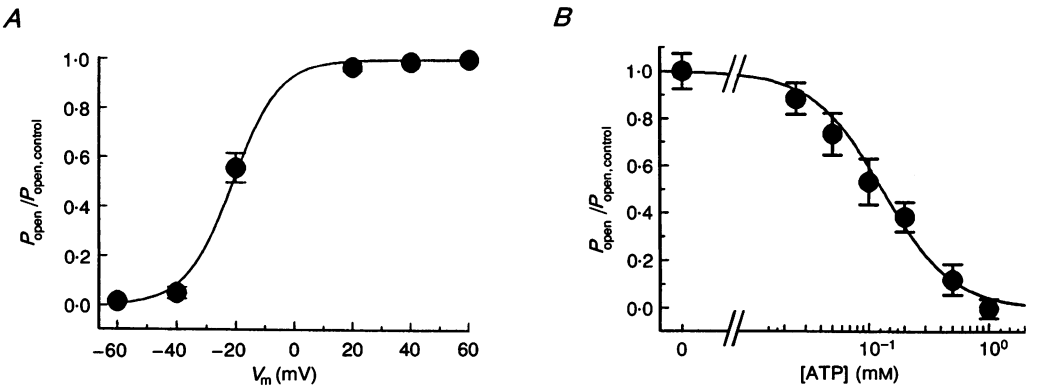


**Figure 4. The large $K^+$ current is voltage-dependent and inhibited by physiological concentrations of ATP**

*A*, the effect of $V_m$ on $P_{open}$. Single-channel currents were recorded from inside-out patches with 0 mM ATP and 1·5 $\mu$M $Ca^{2+}$. When equal concentrations of $K^+$ were applied to both sides of cell membranes (means ± S.E.M., $n = 3$), the channel activity ($P_{open}$, normalized to the maximum level) started to increase at approximately −40 mV. Full activation was reached when $V_m$ was above 20 mV. The relation of $P_{open}$ to $V_m$ can be described with the Boltzmann expression: $P_{open} = 1/\{1 + \exp[(K_v - V_m)/k]\}$, where $P_{open}$ is the open-state probability, $V_m$ the membrane potential, $K_v = -21$ mV ($V_m$ at 50% of $P_{open}$), and $k = 8$ mV. Note that the 0 mV point on the curve is missing, since the potential is very close to the reversal potential. *B*, concentration-dependent inhibition of the open-state probability by ATP. $P_{open}$ was normalized to baseline level ($P_{open}/P_{open,control}$). Data were fitted using the Hill equation: $y = 1/\{1 + ([ATP]/K_d)^h\}$, where $y = P_{open}/P_{open,control}$; [ATP] is the internal ATP concentration. The half-blocking concentration ($K_d$) is 130 $\mu$M and the Hill coefficient ($h$) is 1·5. Data are presented as means ± S.E.M. ($n = 4$). ATP at 50 $\mu$M had a visibly inhibitory effect on channel activity and $P_{open}$ almost reached zero when the ATP concentration was more than 0·5 mM.

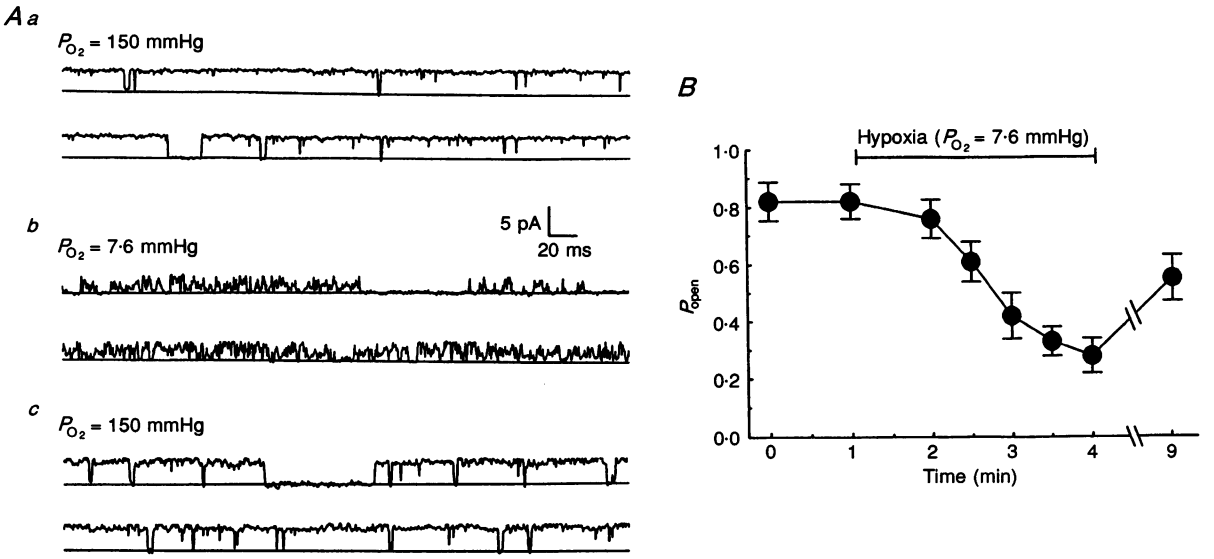


**Figure 5. The large-conductance $K^+$ current is inhibited during hypoxia**

*A*, continuous recordings of a single $K^+$ current from an inside-out patch of a SN neuron, using an equal concentration of $K^+$ (150 mM) on both sides of the membrane with $V_m$ held at 20 mV. During baseline recording (*a*), this channel had a $P_{open}$ of 0·92 and a unitary conductance of 188 pS. Continuous lines indicate the level of channel closing. Hypoxia (~3 min) induced a decrease in $P_{open}$ to 0·24 and a decrease in conductance to 110 pS (*b*). Recovery of both $P_{open}$ (0·96) and conductance (194 pS) is seen after reperfusion (*c*). *B*, time profile of $P_{open}$ during hypoxia. Channel inhibition started about 1 min after the onset of hypoxia and a maximum inhibition was reached in about 3 min. At this level, $P_{open}$ was markedly reduced to about 25% of baseline level. Reperfusion with oxygenated internal medium ($P_{O_2} \approx 150$ mmHg) led to a partial recovery. Data are presented as means ± S.E.M. ($n = 9$). The start time of hypoxia was counted as the time when the hypoxic solution was switched on.

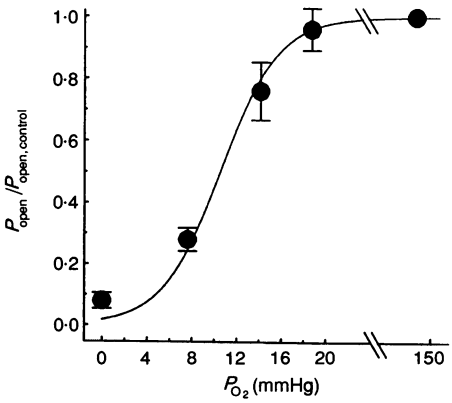


**Figure 6. Dose-dependent inhibition of $P_{open}$ by graded hypoxia**
Inside-out patches were recorded with an equal concentration of $K^+$ (150 mM) on both sides of the membrane. $P_{O_2}$ was measured with polarographic electrodes. Graded hypoxia was induced by perfusing patches with one of the solutions bubbled for at least 2 h with either 2% (balanced with $N_2$), 1% or 0% $O_2$, or 0% $O_2$ plus 2 mM $Na_2S_2O_4$. The measured $P_{O_2}$ from these solutions was 18·7, 14·1, 7·6 and 0 mmHg, respectively. When $P_{O_2}$ was 150 mmHg at baseline, the channel was almost fully open. There was no evident change when $P_{O_2}$ was 18·7 mmHg. Rapid decrease in single-channel $P_{open}$ was seen when $P_{O_2}$ changes between 5 and 15 mmHg. The $P_{O_2}$ for 50% inhibition of $P_{open}$ was 10·5 mmHg. The curve was fitted with the equation $y = 1/\{1 + \exp[(K_D - x)/h]\}$, where $y = P_{open}/P_{open,control}$, $x = P_{O_2}$, $K_D$ is the $P_{O_2}$ level for 50% inhibition of $y$, and $h = 2{\cdot}8$. Note that $P_{open}$ was normalized to its control level. Data are presented as means ± S.E.M. ($n = 11$).

although $P_{open}$ was increased from 0·397 ± 0·122 to 0·500 ± 0·129 (Fig. 8). No inhibitory effect was observed in two other patches that were exposed to anoxia ($P_{O_2} < 1$ mmHg). This strongly suggests that hypoxia selectively inhibits the large $K^+$ channel.

## Single-channel kinetic analysis

The hypoxia-induced inhibition of the large-conductance $K^+$ channel was mediated by a decrease in the mean open time (from 36·1 ± 8·3 to 5·9 ± 1·9 ms, $n = 9$; $P < 0{\cdot}01$) without significantly changing the mean closed time (from 8·3 ± 2·2 to 13·7 ± 3·9 ms, $n = 9$; $P > 0{\cdot}05$). Using open and closed dwell-time histograms from patches that contained only single-channel activity, we found that the open and closed kinetics of this $K^+$ channel were markedly affected by $O_2$ deprivation. Figure 9 shows histograms of the dwell times of the open and closed states of the hypoxia-sensitive $K^+$ channel. The open dwell times were well described by two distinguishable time-constant components ($\tau_{o1} = 4{\cdot}8 \pm 1{\cdot}2$ ms ($w = 29\%$, where $w$ is the fraction of the total fitted area) and $\tau_{o2} = 36{\cdot}4 \pm 5{\cdot}3$ ms ($w = 71\%$), $n = 7$) for each of them when $P_{O_2} = 150$ mmHg (Fig. 9*A*). Both $\tau_{o1}$ and $\tau_{o2}$ decreased markedly when $P_{O_2}$ was reduced to ~8 mmHg (to 1·9 ± 0·7 ($w = 62\%$) and 8·6 ± 3·6 ms ($w = 38\%$), $n = 7$, respectively; Fig. 9*C*). Closed times were described by three distinct time-constant components ($\tau_{c1} = 0{\cdot}5 \pm 0{\cdot}1$ ms ($w = 69\%$), $\tau_{c2} = 4{\cdot}2 \pm 1{\cdot}9$ ms ($w = 25\%$) and $\tau_{c3} = 38{\cdot}4 \pm 21{\cdot}2$ ms ($w = 6\%$), $n = 7$; Fig. 9*B*). The shortest closing time-constant component was about 70% of total events during baseline ($P_{O_2} = 150$ mmHg). During hypoxia ($P_{O_2} = 8$ mmHg), however, this proportion shifted to only about 50%, although the time constants of these three components ($\tau_{c1} = 0{\cdot}7 \pm 0{\cdot}1$ ms ($w = 50\%$), $\tau_{c2} = 3{\cdot}9 \pm 0{\cdot}8$ ms ($w = 42\%$) and $\tau_{c3} = 26{\cdot}9 \pm 7{\cdot}1$ ms ($w = 8\%$), $n = 7$) did not change significantly (Fig. 9*D*). These results indicate that the hypoxia-induced inhibition of the $K^+$ channel activity is mainly due to a decrease in the longer channel openings, resulting in rapid flickerings.

# DISCUSSION

Although major alterations in neuronal function and morphology occur during $O_2$ deprivation, the cascade of events that takes place inside and outside cells, especially the initial steps of that cascade, are not well understood. Most studies centre around the drop in high energy phosphates and its link with a number of cellular events that usually follow. Therefore, one general concept for $O_2$ sensing in neurons (and other cell types) is related to changes in concentrations of cytosolic factors such as ATP. In contrast to this conventional point of view, we show in this paper that neuronal membranes, without the cytosol, can also sense $O_2$ shortage. This challenges currently held concepts regarding $O_2$ sensing in central neurons and the effect of hypoxia on the central nervous system.

At least three observations or ideas gave rise to the question we tried to answer in this work. First, changes in membrane potential or ionic currents start to occur very fast (i.e. within 1 min) when central neurons are exposed to hypoxia (Hansen, Jounsgaard & Hahnsen, 1982; Fujiwara, Higashi, Shimoji & Yoshimura, 1987; Leblond & Krnjević,

1989; Haddad & Donnelly, 1990; Donnelly, Jiang & Haddad, 1992). It is therefore possible that such electrophysiological changes do not result from alterations in cytosolic components, since alterations in factors such as ATP or $Ca^{2+}$ that might be of consequence to cellular function are very likely to take place at least 1–2 min after the start of hypoxia (Kass & Lipton, 1989; Dubinsky & Rothman, 1991; Ekholm, Asplund & Siesjö, 1992; Friedman & Haddad, 1993). Second, recent studies have shown that membrane proteins, including ionic channels, are responsive to changes in their redox state (Ruppersberg, Stocker, Pongs, Heinemann, Frank & Koenen, 1991). This suggests that ionic channel activity can be directly modulated by redox state and therefore by reactions involving the presence or absence of molecular $O_2$. Third, our previous studies have suggested that mechanisms other than those originating in the cytosol may be involved in $O_2$ sensing by nerve cells, since the attenuation in $K^+$ currents after several minutes of $O_2$ deprivation does not seem to be due to channel run-down or to cytosolic ATP- or $Ca^{2+}$-dependent processes (Jiang & Haddad, 1993).

Our present results have shown that $K^+$ channel activity is modulated by $O_2$ deprivation. Since $O_2$ lack inhibits specific $K^+$ channels in excised membranes, it is possible that the observed reduction in whole-cell outward current is due to the decrease in channel activity of this

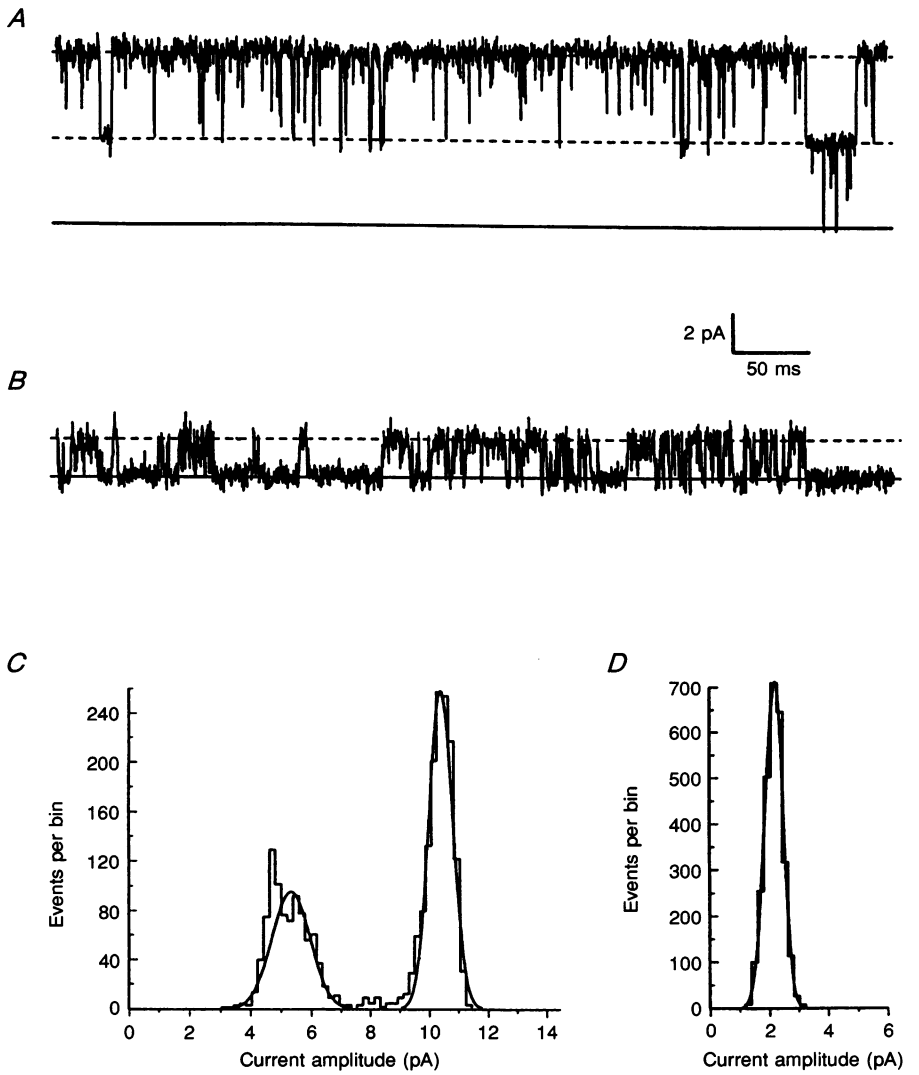


**Figure 7. Current amplitude is inhibited during hypoxia**
Single-channel activity was recorded from an inside-out patch of a SN neuron using an equal concentration of $K^+$ (150 mM) on both sides of the membrane and a $V_m$ of 25 mV. Two active channels (indicated by dashed lines) were seen during baseline (*A*) and both of them had a similar current amplitude (5·1 pA). The continuous line represents the level of channel closing. Note that the filter setting was 0–2 kHz. $O_2$ deprivation for 2·5 min ($P_{O_2} = 7{\cdot}6$ mmHg) induced a marked decrease in single-channel conductance. *C* and *D*, time average of the current amplitude from an 18·5 s period of recording during baseline and hypoxia. The current amplitude was reduced from 5·1 pA during baseline (*C*) to 2 pA during hypoxia (*B*, *D*).

current. Furthermore, since the reduction in these currents is rather rapid (i.e. it occurs in the first few minutes of hypoxia), the initial increase in whole-cell outward currents may be dampened or counteracted by the direct effect of $O_2$ deprivation on neuronal membranes.

The inhibition of $K^+$ channels in neurons by $O_2$ lack seems to be a cytosol-independent process. Although previous studies have shown that cellular organelles such as mitochondria can be associated with certain excised patches (Levitan & Kramer, 1990), we do not believe that the hypoxia-induced inhibition of $K^+$ channel activity is mediated by cytosolic factors. This is because the inhibitory effect of $O_2$ deprivation is just the opposite of the effect induced by changes in ATP and $Ca^{2+}$ concentrations. In addition, several cytosolic factors such as ATP, $Ca^{2+}$ and pH were maintained at constant levels in our experimental solutions after patch excision. Therefore, these factors could not have played a major role in the $K^+$ channel inhibition.

There are at least two potential models that can explain the low-$O_2$-mediated regulation of $K^+$ channel activity. (1) A decrease in $P_{O_2}$ levels depresses the concentration of oxidant species (e.g. $H_2O_2$) which in turn can attenuate $K^+$ channel activity (Cross, Henderson, Jones, Delpiado, Hentschel & Acker, 1990; Kuo, Saad, Koong, Hahn & Giaccia, 1993). (2) $O_2$ deprivation induces a change in the

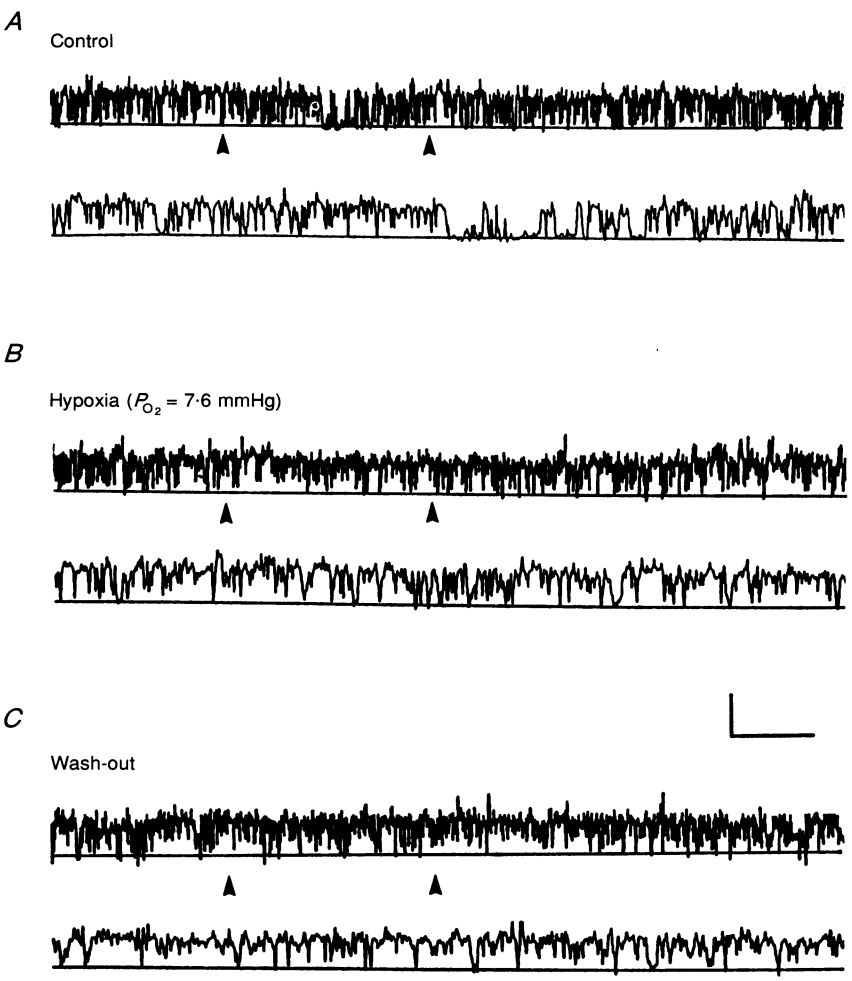


**Figure 8. $O_2$ deprivation does not affect the channel activity of the small-conductance $K^+$ current**

Continuous recordings of a single $K^+$ current from an inside-out patch of a SN neuron, using an equal concentration of $K^+$ (150 mM) on both sides of the membrane with the $V_m$ held at 40 mV. Straight lines indicate channel closing. In each pair of traces, the lower one is taken from the segment between the two arrowheads. During baseline ($P_{O_2}$ = 150 mmHg, *A*), this channel had a $P_{open}$ of 0·79. Hypoxia (~6 min, $P_{O_2}$ = 7·6 mmHg) does not induce an inhibition of the channel activity. Instead, $P_{open}$ increases to 0·91 (*B*). After reperfusion of a medium of $P_{O_2}$ = 150 mmHg, $P_{open}$ remained at the same level as during hypoxia (0·91, *C*). Single-channel conductance (31 pS) is maintained at the same level before, during and after hypoxia. Calibration: 2 pA and 160 ms for the upper trace of each group; 2 pA and 40 ms for the lower one.

channel molecular conformation via a change in channel redox state (Bertl & Slayman, 1990; Ruppersberg *et al.* 1991) or via interactions with other proteins such as metal-containing centres (e.g. iron- or copper-containing proteins). In peripheral chemoreceptive cells such as carotid glomus cells, lack of $O_2$ also induces inhibition of $K^+$ channel activity (López-Barneo, López-López, Ureña & González, 1988; Hescheler, Delpiano, Acker & Pietruschka, 1989; Stea & Nurse, 1991), and is believed to be a membrane-delimited process (Ganfornina & López-Barneo, 1991, 1992). There is increasing evidence that NAD(P)H oxidase, which is present in glomus cells and pulmonary neuroepithelium cell membranes, is a favourite candidate for an $O_2$ sensor (Cross *et al.* 1990; Youngson *et al.* 1993). However, this does not seem to be the case in neurons, since our preliminary data have shown that an NAD(P)H oxidase inhibitor (diphenyliodonium) did not affect channel activity (C. Jiang & G. G. Haddad, unpublished results). Alternatively, other mechanisms may be involved in the $O_2$-sensing phenomenon in central neurons. Metal-containing proteins are present in plasma membranes (Cross *et al.* 1990) and may be associated with channel molecules. When $O_2$ binds to these centres, conformational changes in channel molecules or changes in channel molecular energy state may occur and this can influence channel activity. Another possible mechanism is the redox state of the channel (Bertl & Slayman, 1990; Ruppersberg *et al.* 1991). Changes in the redox state of amino acid residues in channel proteins may also lead to a conformational change and alter channel activity, as has been described recently for A-type $K^+$ currents (Ruppersberg *et al.* 1991). Further experiments are needed to distinguish among these possibilities.

$K^+$ channel regulation during $O_2$ deprivation may have major implications on cell response and adaptation to hypoxic stress. Although the full implications of this novel

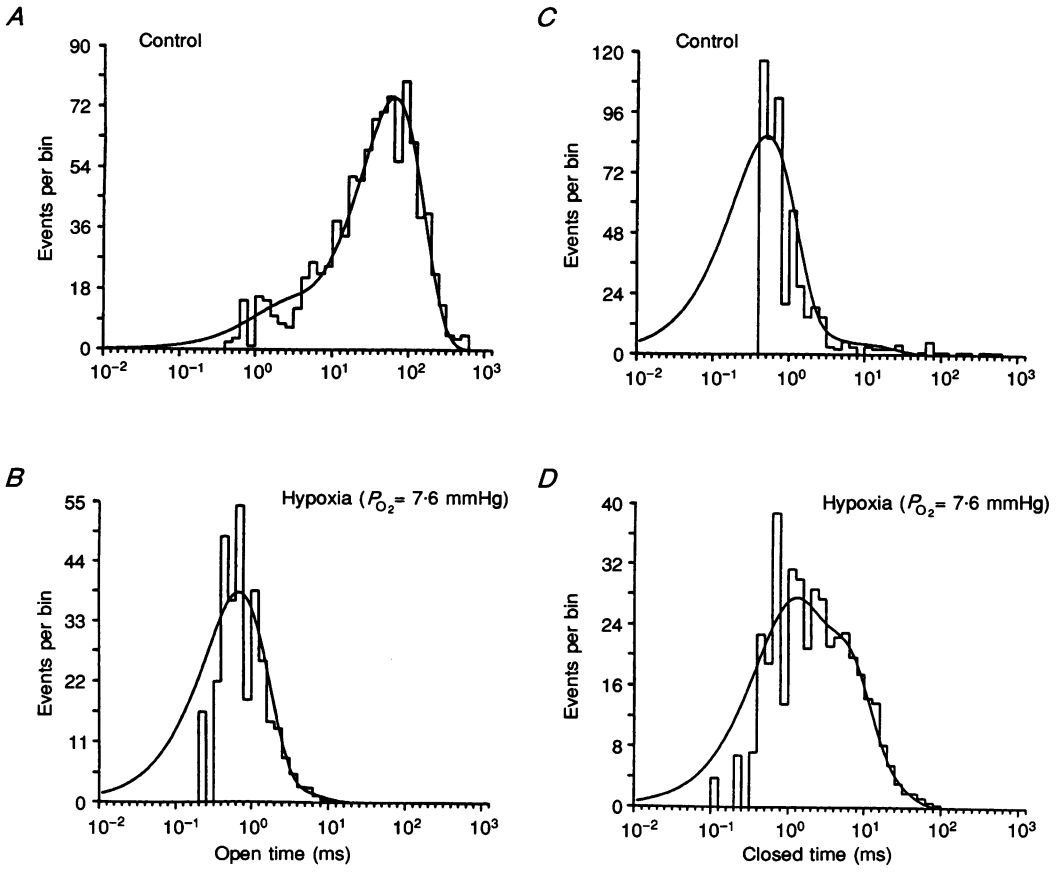


**Figure 9. Single-channel kinetics of open and closed times**
*A*, dwell open-time histogram and the time distribution of events can be well described by a sum of two exponential terms. Two distinct time-constant components are seen with one short time constant ($\tau_{o1}$ = 1·7 ms) and the other much longer ($\tau_{o2}$ = 63·1 ms, total events = 852). The fraction ($w$) of these 2 components is equal to 10% and 90%, respectively. *B*, hypoxia ($P_{O_2}$ = 8 mmHg) suppresses both $\tau_{o1}$ (0·8 ms, $w$ = 95%) and $\tau_{o2}$ (3·3 ms, $w$ = 5%, events = 3112). *C*, dwell closed-time histogram can be fairly fitted by a sum of 3 exponentials ($\tau_{c1}$ = 0·5 ms ($w$ = 89%), $\tau_{c2}$ = 1·7 ms ($w$ = 7%) and $\tau_{c3}$ = 9·8 ms ($w$ = 4%), events = 498). *D*, there is no major change in closed-time components ($\tau_{c1}$ = 0·8 ms, $\tau_{c2}$ = 4·3 ms and $\tau_{c3}$ = 13·2 ms), but the fraction of these components is shifted towards closures of longer time periods ($w_1 : w_2 : w_3$ = 42 : 47 : 11, events = 3443).

mechanism in neurons are not yet clear, a number of potentially important questions and ideas can be raised. First, the cytosol-independent $O_2$-sensing mechanism may allow the cell to respond to its microenvironmental $P_{O_2}$ changes locally. This would enable neurons to regulate their ion channel activity or membrane excitability without altering other cellular functions and ionic homeostasis via changing concentrations of ATP or other cytosolic factors. Second, the same $K^+$ channel can be stimulated by a decrease in intracellular ATP concentration and an increase in $Ca^{2+}$ level, as we have previously demonstrated (Jiang *et al.* 1994). Since changes in concentrations of these cytosolic factors also occur during hypoxia, it is possible that the consequence of $O_2$ deprivation with regard to $K^+$ channel activity depends on the interaction of these cytosolic and membrane factors. While the activation of these $K^+$ channels by a decrease in ATP and increase in $Ca^{2+}$ produces hyperpolarization, the direct effect of $O_2$ deprivation on neuronal membranes is depolarizing in nature. Therefore, the inhibition of $K^+$ channel activity by molecular $O_2$ can counteract some of the effects induced by cytosolic alterations (e.g. ATP and/or $Ca^{2+}$) resulting from lack of $O_2$. These various factors will be important in governing $K^+$ flex and neuronal excitability.

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