

O₂ Modulates Large-Conductance Ca²⁺-Dependent K⁺ Channels of Rat Chemoreceptor Cells by a Membrane-Restricted and CO-Sensitive Mechanism

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Abstract—Hypoxic inhibition of large-conductance Ca²⁺-dependent K⁺ channels (maxiK) of rat carotid body type I cells is a well-established fact. However, the molecular mechanisms of such inhibition and the role of these channels in the process of hypoxic transduction remain unclear. We have examined the mechanisms of interaction of O₂ with maxiK channels exploring the effect of hypoxia on maxiK currents recorded with the whole-cell and the inside-out configuration of the patch-clamp technique. Hypoxia inhibits channel activity both in whole-cell and in excised membrane patches. This effect is strongly voltage- and Ca²⁺-dependent, being maximal at low [Ca²⁺] and low membrane potential. The analysis of single-channel kinetics reveals a gating scheme comprising three open and five closed states. Hypoxia inhibits channel activity increasing the time the channel spends in the longest closed states, an effect that could be explained by a decrease in the Ca²⁺ sensitivity of those closed states. Reducing maxiK channels with dithiothreitol (DTT) increases channel open probability, whereas oxidizing the channels with 2,2'-dithiopyridine (DTDP) has the opposite effect. These results suggest that hypoxic inhibition is not related with a reduction of channel thiol groups. However, CO, a competitive inhibitor of O₂ binding to hemoproteins, fully reverts hypoxic inhibition, both at the whole-cell and the single-channel level. We conclude that O₂ interaction with maxiK channels does not require cytoplasmic mediators. Such interaction could be mediated by a membrane hemoprotein that, as an O₂ sensor, would modulate channel activity. (*Circ Res.* 2001;89:430-436.)

Key Words: hypoxia ■ Ca²⁺-activated K⁺ channels ■ maxiK ■ redox modulation ■ carotid body

Type I cells of the rat carotid body (CB) are chemoreceptors that sense changes in blood pH, CO₂, and O₂. These cells respond to stimulation with a membrane depolarization that induces Ca²⁺ entry through voltage-dependent Ca²⁺ channels and the subsequent release of a neurotransmitter that activates the afferent fibers of the carotid sinus nerve.¹ In the case of hypoxia, the cause of membrane depolarization seems to be the inhibition of K⁺ channels. Three different types of O₂-sensitive K⁺ channels have been postulated to elicit such depolarization: a voltage-gated channel in rabbit type I cells² and a large-conductance Ca²⁺-dependent K⁺ channel (maxiK)³ and a TASK-like channel⁴ in rat type I cells.

Although the role of K⁺ channels in the hypoxic transduction in type I cells is generally accepted, mechanisms involved in the process of O₂ sensing remains unclear.⁵ The prevalent theory to explain hypoxic modulation of K⁺ channels links O₂-sensing to the process of O₂ reduction with some electron donor such as NADPH and postulates that channel activity is modulated by changes in GSH/GSSG or some still undefined reactive oxygen species (ROS).⁶ In fact, oxidizing and reducing agents have been shown to alter channel

activity,⁷⁻¹¹ even in the O₂-sensing channels in type I cells.⁵ In the case of maxiK channels, reducing agents have been reported to increase,^{9,10} decrease,⁸ or not having effect¹² on channel activity. However, although hypoxia and reducing agents could have similar effects on channel activity, they cannot be considered equivalent.¹¹

An alternative hypothesis to explain hypoxic modulation of ion channels proposes the existence of a hemoproteic sensor located in the plasma membrane that modulates channel activity in the absence of soluble factors. Evidence supporting this model has been obtained mainly in rabbit type I cells, where hypoxic modulation of a K⁺ current remains in isolated membrane patches² and can be reverted by CO,¹³ a well-known ligand of hemoproteins. However, there are reports in neonatal rat chemoreceptor cells suggesting that the effect of hypoxia could require some cytosolic factor,^{3,4} although the hypothesis of the hemoproteic sensor has not been directly tested.

In the present study, we investigate the modulation of rat CB maxiK channels by O₂ at the single-channel level. We demonstrate that channel activity is markedly affected by

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changes in O₂ concentration, both in whole-cell and inside-out membrane-excised patches, suggesting that O₂ modulation is a membrane-restricted event. The voltage and Ca²⁺ dependence of O₂ modulation of maxiK channels is compatible with a physiological role of hypoxic inhibition, because the effect was maximal at threshold levels for channel activation. Finally, we show that two redox agents, dithiothreitol (DTT) and 2,2'-dithiopyridine (DTDP), modulate maxiK activity. However, the effect of hypoxia is mimicked by the oxidant, which is what makes the redox hypothesis very unlikely. On the contrary, CO completely reverts the effect of low O₂, as predicted by the hemoproteic sensor hypothesis.

Materials and Methods

Cell Isolation and Culture

Experiments were performed on cultured rat CB chemoreceptor cells. Adult Wistar rats (3 to 4 months old) were obtained from the animal care unit of the University of Valladolid. Rats were anesthetized with pentobarbital sodium (100 mg · kg⁻¹ IP). After tracheostomy, the carotid artery bifurcations were removed, and the animals were killed by an intracardiac bolus injection of sodium pentobarbital. All measures were taken to ensure the animals did not suffer distress at any time. Protocols were approved by the Institutional Animal Care and Use Committee of the University of Valladolid. The CBs were cleaned of surrounding connective tissue and enzymatically dispersed as described elsewhere.¹⁴ Dispersed cells were plated onto small poly-L-lysine-coated coverslips and maintained in culture for up to 48 hours.

Electrophysiological Recordings

Ionic currents were recorded at room temperature (20°C to 25°C) using the whole-cell and inside-out modes of the patch-clamp techniques. Whole-cell currents were recorded and acquired as previously described.¹⁴ Borosilicate glass patch pipettes used for single-channel recordings (2 mm, World Precision Instruments) were double-pulled (Narishige PP-83) and heat-polished (Narishige MF-83) to resistances between 10 and 15 MΩ when filled with the internal solution. Recordings were made with an Axopatch-200A patch-clamp amplifier and a Digidata 1200 A/D interface, driven by CLAMPEX version 8.0 software (Axon Instruments). Single-channel recordings were filtered at 1 to 5 kHz and digitized at 20 kHz.

Analysis

Analysis of the data were performed with pCLAMP software. Single-channel amplitudes and open probabilities were measured from amplitude histograms generated with FETCHAN. Open probabilities were expressed as *NPo*, where *N* represents the number of single channels present in the patch, and *P_o* is the open probability of a single channel. *NPo* was calculated using the following expression:

$$(1) \quad NPo = (A_1 + 2 \cdot A_2 + 3 \cdot A_3 + \dots + n \cdot A_n) / (A_0 + A_1 + A_2 + \dots + A_n)$$

where *A*₀ is the area under the curve of the amplitude histogram corresponding to the closed state, and *A*₁–*A*_{*n*} represent the histogram areas reflecting the different open-state current levels for 1 to *n* channels present in the patch. Histogram parameters were obtained from multiple least-squares gaussian fits of the data using ORIGIN 4.0 software (Microcal Inc).

Single-channel kinetic analysis was performed using the QuB Suite package (New York State University, Buffalo, NY). Single-channel traces were idealized with SKM, and model fitting to data were carried out with MIL.^{15,16}

Pooled data are expressed as mean ± SEM. Statistical comparisons were performed with the two-tailed *t* test for paired or unpaired data

as appropriate. Values of *P* < 0.05 were considered statistically different.

Solutions

The composition (in mmol/L) of the bathing solution used for gigaseal forming and whole-cell recordings was NaCl 141, KCl 4.7, MgCl₂ 1.2, CaCl₂ 1.8, glucose 10, and HEPES 10 (adjusted to pH 7.4 with NaOH). Whole-cell experiments were performed with a pipette solution containing (in mmol/L) KCl 125, MgCl₂ 4, EGTA 10, MgATP 5, NaGTP 5, and HEPES 10 (adjusted to pH 7.2 with KOH). Inside-out recordings were carried out with a pipette solution containing (in mmol/L) KCl 125, EGTA 5, and HEPES 10 (adjusted to pH 7.2 with KOH). After patch excision, the bath solution was switched to a solution of identical composition to that of the pipette but containing different amounts of CaCl₂ to get free [Ca²⁺] of 1, 5.5, or 10 μmol/L. To obtain 100 μmol/L free Ca²⁺, HEDTA instead of EGTA was used as Ca²⁺ buffer. Total Ca²⁺ was calculated using the program CHELATOR.¹⁷ Titrated stock solutions of CaCl₂ and EGTA (HEDTA) were used to minimize errors due to impurities of EGTA (HEDTA) and hydration of CaCl₂.

Bathing solutions were perfused by gravity into the recording chamber at 1 to 2 mL/min. Control conditions are defined as the O₂ level present in solutions equilibrated with air (150 mm Hg). Hypoxia was achieved by bubbling the bath solution with 100% N₂, giving a P_{O₂} in the recording chamber of 9.5 ± 1.2 mm Hg (*n* = 5), as determined with an O₂ microelectrode (Diamond General Corp). O₂ electrode was calibrated using solutions equilibrated with air (150 mm Hg) or containing 1 mmol/L sodium dithionite (Na₂S₂O₄) as an O₂ scavenger (0 mm Hg). When CO was used, the bathing solution was bubbled with a mixture of 80% N₂ and 20% CO, that gives a P_{O₂} of 8.7 ± 2.3 mm Hg (*n* = 5). In selected experiments, control solution was also bubbled with air to avoid possible artifacts introduced in the record by bubbling. No differences were found between these two control groups.

Results

O₂ Sensitivity of MaxiK Channels in Isolated Patches

MaxiK channel activity was recorded from inside-out patches. Patches with several (typically 2 to 5) large conductance channels (186 ± 12 pS, in symmetrical K⁺, *n* = 21) were routinely obtained. In most cases, the activity of these channels was completely abolished on removal of Ca²⁺ from the bath solution. Patches with channels of smaller conductance and/or Ca²⁺-independent were not considered for analysis. A typical experiment is shown in Figure 1. Figure 1A represents a diary plot of channel activity in the patch. This plot was built by computing the *NPo* in 5-second intervals and representing the obtained value against recording time. The activity of three channels was evident in control conditions, as shown in the sample current trace (Figure 1A, 1). When hypoxia was applied, channel activity decreased, and only two channels opened simultaneously (Figure 1A, 2). The channel activity recovered after returning to control solution (Figure 1A, 3). Removing Ca²⁺ from the bath solution completely abolished channel activity in the patch. The all-point histograms corresponding to periods of 2 minutes in control (1), hypoxia (2), and recovery (3) conditions are shown in Figure 1B. Average *NPo* calculated from these histograms were 0.73, 0.32, and 0.75, respectively. Similar results have been obtained in 49 patches of 65 tested.

Voltage and Ca²⁺ Dependence of the Effect of Hypoxia

Figure 2 shows the effect of membrane potential on the open probability of a single maxiK channel when [Ca²⁺]_i was kept

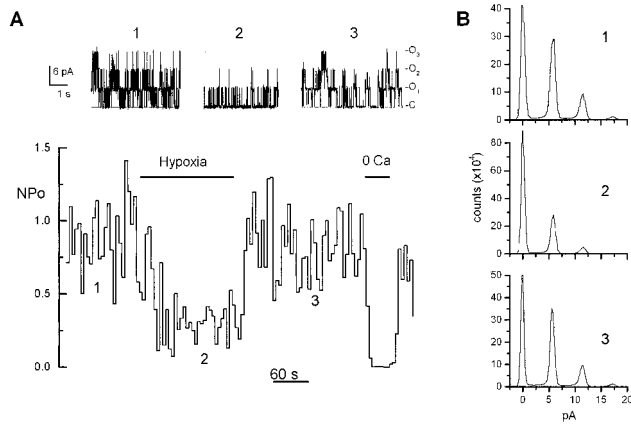


Figure 1. Effect of hypoxia on maxiK channel activity in an inside-out excised membrane patch. A, Graph (bottom) showing the effect of hypoxia and 0 Ca²⁺ on open probability (NPo) of maxiK channels. Representative current traces (top) recorded before (1), during (2), and after (3) exposure to hypoxic solution are shown. Holding potential, +30 mV. Free [Ca²⁺], 1 μmol/L. B, All-point histograms corresponding to periods of 2 minutes before (1), during (2), and after (3) hypoxic perfusion.

at 10 μmol/L. The increase of open probability on depolarization is well described by a Boltzmann equation (Figure 2B, filled squares). Hypoxia (empty circles) decreased the open probability of the channel by shifting the voltage curve 24.6 mV to the right. As expected from the Boltzmann functions, when expressed as percentage of inhibition of open probability (open triangles), the effect of hypoxia is maximal at lower potentials (around the activation threshold) and decreases sharply with depolarization. Similar results were obtained in two additional patches (average shift: 26.8±5.1 mV).

The effect of hypoxia on maxiK channels was also investigated at different [Ca²⁺] (Figure 3). Figure 3A shows the all-point histograms obtained in a single-channel patch with

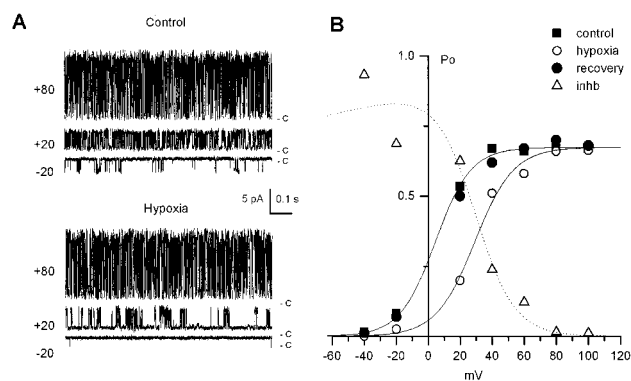


Figure 2. Voltage dependence of the effect of hypoxia on maxiK activity. A, Representative single-channel currents recorded at -20, +20, and +80 mV in control conditions (top) and after exposure to a hypoxic solution (bottom). B, Po versus voltage graph obtained in control conditions (■), during exposure to a hypoxic solution (○), and after returning to control conditions (●). Continuous lines are fits with the Boltzmann equation $Po = A / \{1 + \exp[(V - V_{0.5})/dV]\}$, where A was fixed to 0.67, the maximal Po observed in this channel. Values of V_{0.5} and dV are 4.3 and 11.2 for control and 28.9 and 12.2 for hypoxia. Open triangles (△) represent the effect of hypoxia on Po calculated as $inhb = 1 - (Po_{hypoxia}/Po_{control})$. Dotted line represents the same calculation but using Po values from the Boltzmann fits.

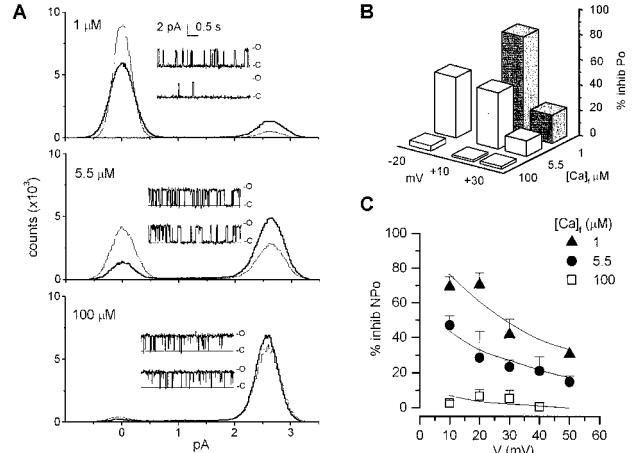


Figure 3. Ca²⁺ dependence of the effect of hypoxia on maxiK activity. A, All-point histograms corresponding to 2-minute periods of channel activity under control (thick lines) or hypoxic (thin lines) conditions at 1 μmol/L (top), 5.5 μmol/L (middle), and 100 μmol/L Ca²⁺ (bottom). Holding potential was +10 mV. Sample current traces obtained in each condition are depicted as insets. In each panel, upper and lower traces were recorded under control and hypoxic conditions, respectively. B, Hypoxic inhibition of Po ($\%inhb = 100 - (100 \cdot Po_{hypoxia}/Po_{control})$) obtained at 3 different potentials and 3 different free [Ca²⁺] in the same patch depicted in panel A. C, Effect of hypoxia expressed as percentage of inhibition of NPo is represented against holding potential at 3 different free [Ca²⁺] (1 [▲], 5.5 [●], and 100 μmol/L [□]). Data are mean±SEM of measures obtained in 4 to 9 different patches.

holding potential of +10 mV at three different [Ca²⁺], both in control and hypoxic conditions. Insets show sample current traces obtained in each situation. Channel activity increased by raising [Ca²⁺] (top to bottom), but this condition concurrently decreased the effect of hypoxia. At 1 μmol/L Ca²⁺ hypoxia inhibited the channel activity by ≈70%, but this effect was ≈46% at 5.5 μmol/L and only 3% at 100 μmol/L. The same three [Ca²⁺] were also tested in this channel at -20 and +30 mV (Figure 3B). Patch depolarization decreased the low O₂-induced inhibition, as expected from the above-described voltage dependence of this effect. The effect of hypoxia was tested at different voltages and [Ca²⁺] in 9 additional patches, although not all voltages and [Ca²⁺] were checked in every experiment. Average data, expressed as percentage of inhibition of NPo, are represented against membrane potential at three different [Ca²⁺] in Figure 3C. Hypoxic inhibition was maximal at lower potentials and lower Ca²⁺ concentrations.

Effects of Low PO₂ on Channel Kinetics

The effect of hypoxia on channel kinetics was analyzed in detail in 4 single-channel patches. The Po measured in one of those patches is shown in Figure 4A. The kinetic behavior of this channel was investigated computing the stability plots depicted in Figure 4B. These plots represent the mean duration of groups of 50 sequential open and closed intervals plotted against the sequential interval number. Inspection of these plots reveals that the kinetic behavior of the channel remains constant over time, and that lowering the PO₂ increases the duration of the closed intervals. This effect

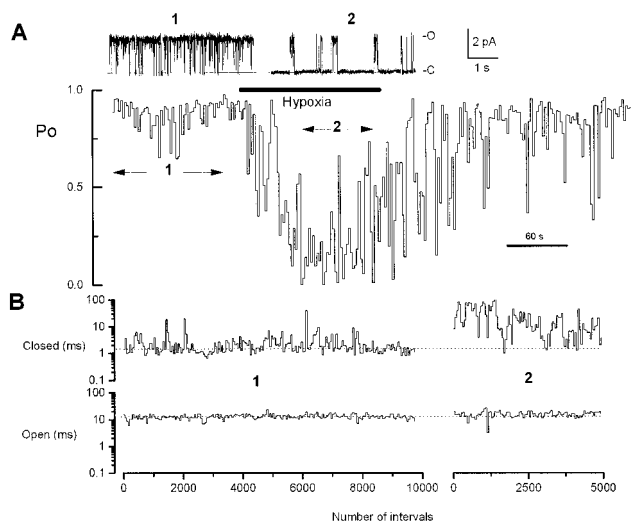


Figure 4. Effect of hypoxia on maxiK channel kinetics. A, Graph showing the effect of applying hypoxic solution to an excised patch containing a single maxiK channel. P_o computed in 2-second intervals is represented against time. Top, Insets show representative current traces obtained before (1) and during (2) application of the hypoxic solution. B, Stability plots of the average durations of successive closed and open intervals before (left) and during (right) exposure to the hypoxic solution. Average intervals correspond to the segments labeled 1 and 2 in panel A. Closed and open intervals were separated, and groups of 50 were averaged and represented against the sequential interval number. Holding potential was +10 mV and free $[Ca^{2+}]$ was 5.5 $\mu\text{mol/L}$.

becomes more evident when the dwell-time histograms corresponding to control and hypoxic conditions are represented superimposed (Figure 5). Whereas the open-time histograms are almost identical, the closed-time histograms markedly differ in the number of counts corresponding to the longer closed durations.

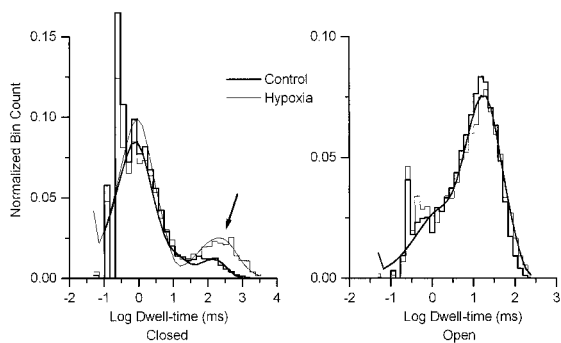
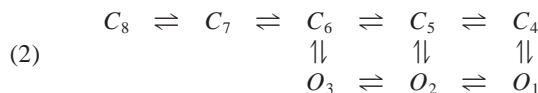


Figure 5. Kinetics of the inhibition of maxiK channels by hypoxia. Closed- and open-dwell time histograms obtained in control conditions (thick lines) and during application of a hypoxic solution (thin lines). The smooth curves are the theoretical probability density functions calculated from the fitted rate constants to the scheme depicted in the text. The values of the rate constants were (s^{-1}) as follows: k_{12} 33.66 ± 2.44 ; k_{21} 6.77 ± 1.26 ; k_{41} 1.26 ± 0.13 ; k_{14} 6.31 ± 0.70 ; k_{52} 1804.69 ± 156.84 ; k_{25} 37.77 ± 3.30 ; k_{32} 940.78 ± 79.34 ; k_{23} 94.79 ± 11.43 ; k_{54} 76.57 ± 11.55 ; k_{45} 1.59 ± 0.15 ; k_{63} 810.89 ± 70.75 ; k_{36} 477.36 ± 43.57 ; k_{76}/h 546.56 ± 50.82 ; k_{67} 619.46 ± 57.18 ; k_{87}/h 33.50 ± 1.49 ; k_{78} 154.01 ± 9.61 ; k_{65} 101.84 ± 36.18 ; and k_{56} 288.66 ± 78.73 . Rate constants were identical in control and hypoxia except k_{76} and k_{87} , which were made O₂-dependent by dividing their values by 2.8 (h) in hypoxic conditions.

Open-time histograms were properly described in all single-channel patches by the sum of 3 exponential functions, whereas closed-time histograms required 4 or 5. The number of states suggested by this analysis is compatible with the gating scheme proposed by McManus and Magleby¹⁸ to describe the kinetics of maxiK channels from rat skeletal muscle.



To test if this model also fits the data obtained in the present work, we used the program MIL, which computes the likelihood of the experimental sequence of open and closed times given a set of trial rate constants and changes the rate constants to maximize the likelihood. Proper constraints were introduced during the fitting procedures to ensure the microscopic reversibility of the loops in the model. The program also allows to fit simultaneously sets of data obtained under different experimental conditions. Therefore, data under control and hypoxic conditions were fitted simultaneously, making O₂-sensitive different rate constants of the scheme. Because the effect of hypoxia was in all cases an increase in the duration of the longest closed states without changing the open states, only the forward transitions between the closed states were considered O₂-dependent during the fit. In the experiment shown in Figure 4, the maximum likelihood was obtained when the forward rate constants of the transitions between C₈-C₇ and C₇-C₆ were made O₂-dependent. The best fit was obtained when the effect of hypoxia on these rate constants was to decrease their value by a factor (h) of 2.8. The dwell-time histograms and the probability-density functions derived from the fitted parameters are shown in Figure 5. Results obtained in another two channels were also well fitted by this model, with h averaging 3.5 ± 0.85 ($n=3$).

Effect of Redox Agents on Channel Activity

One possible mechanism to explain the effect of hypoxia could be the redox modulation of the cysteine sulfhydryl residues of the channel. In that case, the reducing environment created by hypoxia should be mimicked by a reducing agent such as DTT and blocked by an oxidant such as DTDP. The effect of DTT on channel activity was explored in five different inside-out patches containing 2 to 4 maxiK channels. Figure 6A shows representative current traces corresponding to one of those patches. DTT increased the activity of the channels in the patch; this effect was reversible on returning to control solution. The effect of DTDP has been also explored in 5 additional patches. Average results obtained at different holding potentials and expressed as percentage of NPo in control conditions are also depicted in Figure 6B.

The interaction between redox and O₂ modulation has been further investigated using the whole-cell configuration of the patch-clamp technique. Cells were voltage-clamped at -80 mV, and depolarizing pulses to +20 mV were applied at 0.05 Hz. Figure 7A shows the plot of peak current versus time of pulse application obtained in a typical experiment. Peak current slowly runs down along the experiment, because of

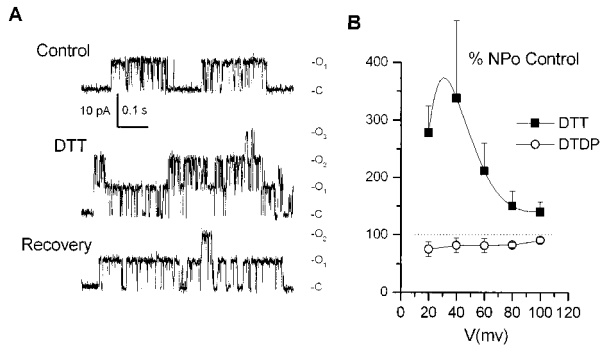


Figure 6. Effect of DTT and DTDP on maxiK channel activity in inside-out patches. A, Representative current traces obtained in a membrane patch containing 3 maxiK channels before (NPo 0.56), during (NPo 1.2), and after (NPo 0.7) application of 100 $\mu\text{mol/L}$ DTT. Holding potential, +40 mV. Free $[\text{Ca}^{2+}]$, 5.5 $\mu\text{mol/L}$. B, Effect of DTT (100 $\mu\text{mol/L}$) and DTDP (20 $\mu\text{mol/L}$) on channel activity expressed as percentage of NPo in control conditions ($\%NPo_{\text{control}} = 100 \cdot NPo_{\text{DTT(DTDP)}/Po_{\text{control}}}$) and represented against membrane potential. Data are mean \pm SEM, n=5.

the washout of Ca^{2+} channels. The contribution of maxiK channels to the whole K^+ current can be estimated by inhibiting Ca^{2+} entry during the pulse with Cd^{2+} at the end of the experiment. Both hypoxia and DTDP produced an inhibition of K^+ current, as expected from the single-channel data shown in the previous figures. Interestingly, the effect of hypoxia was not affected by the presence of DTDP, suggesting different mechanisms for both stimuli. The effect of DTDP was almost irreversible (data not shown), but fully reverted with the application of DTT. Average results obtained in 5 different cells are shown in Figure 7B. DTT data correspond to 4 cells where this agent was applied without previous application of DTDP.

Reversion of Hypoxic Inhibition by CO

Alternatively to the redox mechanism, O_2 could interact with a sensor in the plasma membrane just as a ligand, so that its degree of saturation would modulate channel activity. In this hypothesis, the effect of hypoxia should revert after saturating the sensor with an alternative ligand. If the sensor were a hemoprotein, a putative ligand would be CO. Therefore, we have investigated the effect of CO on the inhibition of channel activity by hypoxia. First, we have studied the effect of CO using a protocol similar to that in Figure 7A. Figure 7C shows a typical experiment. When hypoxia was applied to the bath solution, peak current decreased by $\approx 30\%$, and this inhibition was almost fully reverted adding 20% CO to the hypoxic solution. When the effect of hypoxia is measured on the Cd^{2+} -sensitive current, inhibition increased to $\approx 50\%$. Average results obtained in 15 different cells are shown in Figure 7D.

The effect of CO on the hypoxic response was also tested in isolated patches in the inside-out configuration. Figure 8A shows the NPo diary plot of a patch containing at least 3 maxiK channels. In this experiment, hypoxia decreased NPo by $\approx 50\%$, and adding CO to the hypoxic solution not only reverted the effect of hypoxia but increased NPo to $\approx 150\%$. Current traces corresponding to control conditions, hypoxia, and hypoxia plus CO are depicted below the diary plot.

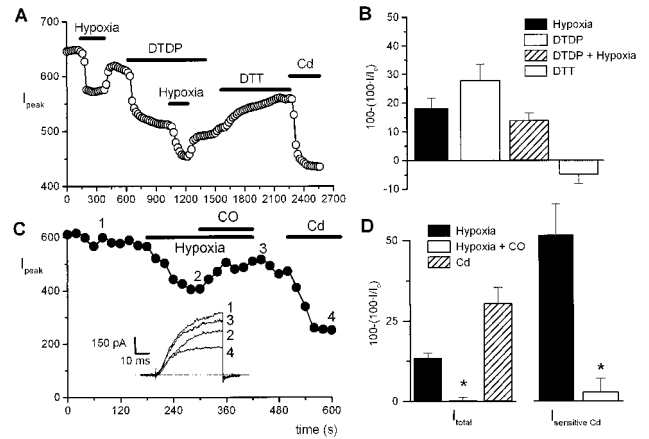


Figure 7. Effect of redox agents and CO on hypoxic response of whole-cell K^+ currents. A, Peak currents elicited by 50-ms depolarizing pulses from -80 to $+20$ mV applied every 20 seconds are plotted against time of pulse application. Hypoxia, DTDP 20 $\mu\text{mol/L}$, DTT 100 $\mu\text{mol/L}$, or Cd^{2+} 100 $\mu\text{mol/L}$ were applied as indicated. B, Average results obtained in 5 experiments with the protocol shown in panel A. DTT data were obtained in 4 cells without the previous application of DTDP. Data were expressed as $\%Inhibition = 100 - 100 \cdot I_{\text{stimul}}/I_{\text{control}}$. In the case of hypoxia plus DTDP, the calculation was taken as $\%Inhibition = 100 - 100 \cdot (I_{\text{DTDP}} - I_{\text{hypoxia}})/I_{\text{control}}$. C, Whole-cell peak currents obtained in a cell following a protocol similar to that of panel A. Stimuli were applied as indicated. Representative traces corresponding to pulses applied in control conditions (1), during application of hypoxia (2), hypoxia plus 20% CO (3), and 100 $\mu\text{mol/L}$ Cd^{2+} (4) are shown in the inset. D, Effect of hypoxia or hypoxia plus CO expressed as percentage of inhibition of control current, either considering as control the total current or the Cd^{2+} -sensitive. Results are mean \pm SEM, n=14. $*P < 0.01$.

Similar experiments were performed in 4 additional patches at the same $[\text{Ca}^{2+}]$ and at 3 different potentials. In all cases, CO reverted the effect of hypoxia. Average results are shown in Figure 8B.

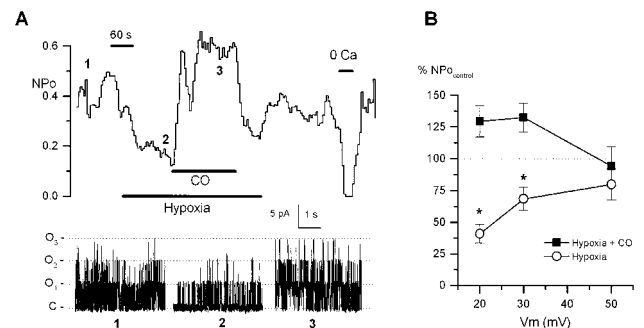


Figure 8. Effect of CO on hypoxic response of maxiK channels in inside-out membrane patches. A, NPo measured in 5-second intervals in a patch containing 3 maxiK channels (numbered 1, 2, and 3 on the graph) is represented against time. Hypoxia, CO, and 0 Ca^{2+} were applied when indicated by the horizontal bars. Bottom, Representative records corresponding to perfusion of the patch with control solution (1), hypoxia (2), and hypoxia plus 20% CO (3). Holding potential was +30 mV and free $[\text{Ca}^{2+}]$ was 10 $\mu\text{mol/L}$. B, Average results obtained at 3 different potentials in 3 different patches. Free $[\text{Ca}^{2+}]$ was 10 $\mu\text{mol/L}$. The effect of hypoxia (\circ) and the effect of hypoxia plus CO (\blacksquare) on channel activity were expressed as percentage of NPo control ($\%NPo_{\text{control}} = 100 \cdot NPo_{\text{stimulus}}/Po_{\text{control}}$). $*P < 0.01$.

Discussion

The present study demonstrates a membrane-delimited mechanism for hypoxic modulation of maxiK channels of rat CB type I cells. The [Ca²⁺] and voltage dependence of this effect are consistent with a physiological role for maxiK channels in the process of hypoxic transduction. Furthermore, our results provide some insights into the mechanisms of O₂ modulation of maxiK channels, suggesting the existence of a hemoproteic sensor located in the plasma membrane.

O₂ modulation has been described for many different ion channels, and not only in classical chemoreceptor cells.^{5,19} However, very little is known about the molecular mechanisms of such modulation. Data presented in this study clearly show that interaction of O₂ and maxiK channels can occur through a membrane-delimited mechanism, because hypoxic modulation occurs in excised membrane patches, in the absence of cytoplasmic factors. Although we cannot exclude the involvement of soluble factors in the modulation of the response to hypoxia, certainly such factors are not a requirement *sine qua non* for the response. Membrane-delimited mechanisms for O₂ modulation of different K⁺ channels have been proposed both in native cells^{20–22} and in heterologous expression systems.^{11,22} Cloned human brain maxiK channels stably expressed in HEK293 have also been reported to be modulated by hypoxia through some direct mechanism.²³ However, the requirement of cytosolic factors has been postulated in the case of maxiK channels of neocortical neurons from young mice²⁴ or neonatal rat type I cells.³ Although we do not have an explanation for the discrepancy between the latter findings and ours, the fact that the studies of Wyatt and Peers³ were carried out in neonatal cell preparations makes conceivable the hypothesis that the mechanism of O₂ detection can change during postnatal development.

The effect of [Ca²⁺] and voltage on maxiK kinetics has been extensively studied in different preparations,^{18,25} and a kinetic scheme comprising 3 open and 5 closed states has been proved to explain many properties of maxiK channels, although more complicated extensions of the model are needed to fully characterize channel properties.²⁶ In spite of some discrepancies, Ca²⁺ and voltage seem to activate maxiK channels increasing mainly the forward rate constants between closed (C-C) and open (O-O) states. Although we have not performed a detailed study of the Ca²⁺ and voltage dependence of type I cell maxiK channels, our results are consistent with such effects (data not shown). Hypoxia increases longer closed durations without changing significantly the shape of the open-dwell time histograms. Taking into account the pronounced influence of [Ca²⁺] on the hypoxic modulation of the channel, O₂ could affect the Ca²⁺ sensitivity of maxiK channels. If we assume that the forward rate constants between closed (C-C) and open (O-O) states are Ca²⁺-dependent, as reported for other preparations, hypoxia would be modifying the rate constants of forward transitions between the longest closed states, C₈, C₇, and C₆. This assumption provides a reasonable fit of the data when activity of the channel in control and hypoxic conditions is fitted simultaneously. The model also predicts a larger effect of hypoxia at lower [Ca²⁺] and lower potentials, when the

channel spends more time in the closed states. To date, apart of this study, the only attempt of investigating the effects of lowering O₂ in the gating properties of a K⁺ channel has been the work of Ganfornina and López-Barneo² on rabbit chemoreceptor cells. In spite of differences in the nature of the K⁺ channels in both preparations (maxiK versus voltage-dependent), it is remarkable that in both cases hypoxia modulates open probability, stabilizing the closed states of the channels.

O₂ could interact directly with the channel molecule or, alternatively, with a putative O₂ sensor that would modulate channel activity. K⁺ channels have cysteine and methionine residues susceptible of redox modulation,²⁷ and that property has been related in some preparations with their hypoxic sensitivity. In this hypothesis, O₂ sensing is linked to the process of O₂ reduction with some electron donor. Changes in ROS production would modulate channel activity, directly or through changes in GSH/GSSG. Our results are clearly not compatible with such a mechanism. The effect of O₂ in excised inside-out patches excludes both the requirement of an electron donor and the possible role of a soluble molecule. Furthermore, the reversibility of the effect under these cell-free conditions rules out the reduction by hypoxia of methionine residues of the channel as a possible mechanism.²⁷ In addition, application of the reducing agent DTT produces an increase of maxiK channel activity, an effect exactly opposite to that of hypoxia. Furthermore, application of the oxidant agent DTDP decreases channel activity, although the effect of hypoxia is not affected by the presence of the oxidant. Moreover, whereas the redox state of the channels in whole-cell and excised patches varies,¹⁰ as evidenced by the effects of DTT and DTDP, hypoxic inhibition remains essentially unchanged in the two environments. Taken together, the data indicate that reduction of channel cysteine or methionine residues cannot account for the effect of hypoxia. By contrast, data provided in this study suggest the presence in the plasma membrane of a hemoproteic sensor, capable of binding both O₂ and CO. This sensor would inhibit channel activity when desaturated. As predicted, CO reverts the hypoxic inhibition of maxiK channel activity. In fact, this activity is slightly larger when solutions are equilibrated with 20% CO than with air (20% O₂), suggesting that the sensor could have a higher affinity for CO. A hemoproteic sensor has already been postulated to explain hypoxic transduction in rabbit chemoreceptor cells,¹³ pulmonary artery vasoconstriction,²⁸ or the effect of hypoxia on cloned channels expressed in HEK293.¹¹

The role of maxiK channels in type I cells of rat CBs in response to hypoxia is not clear in the literature.^{3,29,30} In order to participate in the depolarization triggered by hypoxia, maxiK channels should contribute to the resting membrane potential; if that were not the case, these channels would just act as negative-feedback regulators of membrane potential and [Ca²⁺]_i changes elicited by activation of chemoreceptor cells, and O₂ modulation of maxiK channels would contribute to control such regulation. In the present study, we do not attempt to solve this controversy, although data regarding the voltage and Ca²⁺ dependence of hypoxic modulation of maxiK channels can provide some insights in both possible scenarios. Hypoxia produces a shift to the right of the voltage dependence curve of maxiK channels, leading to an inhibition

of channel activity at lower potentials. If some maxiK channels are open at resting potentials, hypoxia could lead to membrane depolarization. If they are not open, hypoxic membrane depolarization should be just a consequence of TASK channel inhibition.⁴ In this case, inhibition of maxiK channels at the initial states of such depolarization should prevent a prompt repolarization induced by the activation of maxiK by Ca^{2+} and depolarization. Hypoxic inhibition of maxiK channels would be released at higher potentials or higher $[\text{Ca}^{2+}]_i$, allowing full maxiK channel activation. As a consequence, inhibition of maxiK channels would amplify the cell response to hypoxia without losing the negative-feedback role of the channels.

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