Post-transcriptional Control of Human maxiK Potassium Channel Activity and Acute Oxygen Sensitivity by Chronic Hypoxia*

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Various cardiorespiratory diseases (e.g. congestive heart failure, emphysema) result in systemic hypoxia and patients consequently demonstrate adaptive cellular responses which predispose them to conditions such as pulmonary hypertension and stroke. Central to many affected excitable tissues is activity of large conductance Ca2+-activated K+ (maxiK) channels. We have studied maxiK channel activity in HEK293 cells stably co-expressing the most widely distributed of the human α- and β-subunits that constitute these channel following maneuvers which mimic severe hypoxia. At all [Ca2+]i, chronic hypoxia (18 mm Hg, 72 h) increased K+ current density, most markedly at physiological [Ca2+]i, K+ currents in cells cultured in normoxia showed a [Ca2+]i-dependent sensitivity to acute hypoxic inhibition (25 mm Hg, 3 min). However, chronic hypoxia dramatically changed the Ca2+-sensitivity of this acute hypoxic inhibitory profile such that low [Ca2+]i could sustain an acute hypoxic inhibitory response. Chronic hypoxia caused no change in α-subunit immunoreactivity with Western blotting but evoked a 3-fold increase in β-subunit expression. These observations were fully supported by immunocytochemistry, which also suggested that chronic hypoxia augmented α/β-subunit colocalization at the plasma membrane. Using a novel nuclear run-on assay and RNase protection we found that chronic hypoxia did not alter mRNA production rates or steady-state levels, which suggests that this important environmental cue modulates maxiK channel function via post-transcriptional mechanisms.

Crucial to the cellular and physiological response to acute perturbation of systemic and/or pulmonary O2 levels is the rapid inhibition of K+ channels by hypoxia (see Ref. 1 for recent review). Thus, acute modulation of ion channel activity is central to the homeostatic mechanisms that underlie chemosensing in carotid body (2–4), neuroepithelial body (5, 6) (and its immortalized cellular counterpart, H146 cells, Ref. 7) and (8–11) systemic vascular smooth muscle (12). Although somewhat controversial, ion channel inhibition has also been implicated in hypoxic vasoconstriction in the pulmonary circulation (13).

In addition, such O2 sensitivity is believed to play a significant role in modulation of excitability in several cellular components of the mammalian nervous system (14–17).

Although O2-sensitive tissues express a wide variety of channel types, central to the cellular mechanism of acute O2 sensing in several is hypoxic suppression of large conductance Ca2+-activated K+ (maxiK) channels. Indeed, hypoxic inhibition of native maxiK channel activity has been demonstrated in carotid body (4, 18, 19), pulmonary arteriolar smooth muscle (20), chromaffin cells (21), and central neurons (20, 22). Although their contribution to carotid body, chromaffin cell, and central neuronal function is well supported, some controversy still surrounds their involvement in pulmonary vasoconstriction (15) and there is good evidence for both delayed rectifier (23) and tandem P domain K+ channels in the response (24); the latter observation is fully supported by our recent reports of O2 sensitivity of the recombinant human tandem P domain channels, hTASK1 (25), and hTASK3 (26).

Tissue specificity notwithstanding, we have recently demonstrated at the single channel level that a recombinant human maxiK channel can be rapidly and reversibly inhibited by acute hypoxia; this inhibition is underlain by hypoxia-evoked depression in unitary conductance, slowed channel activation kinetics (without an effect on channel deactivation kinetics), reduced open-state probability, and altered channel sensitivity to intracellular calcium concentration ([Ca2+]i) (27). Although it has been suggested that the mechanism of hypoxic regulation of K+ channels in general, and maxiK channels in particular, may involve O2-dependent modulation of cellular redox potential, the nature of the sensor is still unclear and the mechanism whereby decreased pO2 evokes inhibition of this channel type in different tissues remains controversial. Indeed, there are reports that have suggested involvement of either cytolic factors (e.g. Refs. 18 and 20) or more direct channel modulation (e.g. Refs. 17 and 19). In favor of potential regulation by cellular redox state are recent data demonstrating activation of these channels by oxidizing agents in a recombinant cellular system (28) and inhibition by reduced glutathione in rat hippocampal neurons (29). However, we have shown that channel inhibition by hypoxia is maintained in excised inside patches from HEK293 cells stably expressing the human α/β-maxiK channel (27). Consequently, the likelihood of an interaction of soluble cytolic components with this particular channel accounting for hypoxic inhibition seems remote.

Individuals suffering from a variety of cardiorespiratory diseases (such as chronic obstructive pulmonary disease, apnea of sleep, emphysema, congestive heart failure, and stroke) un-

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Chronic Hypoxic Regulation of maxiK Channel Function

Detergo adaptive responses. Given the central importance of K⁺ channels to cellular function, it seems likely that remodeling of channel functional activity by sustained or intermittent episodes of hypoxia may contribute to certain pathologies such as pulmonary hypertension (see Refs. 30–32 for recent reviews) in addition to the well documented adaptation to high altitude (33). Such a notion is supported by observations in exemplar chemosensory tissues such as the pulmonary vasculature (where voltage-gated K⁺ channel expression is selectively suppressed in chronic hypoxia, Ref. 34) and the carotid body (where chronic hypoxia both in vivo, Refs. 35 and 36 and in vitro Refs. 37–39 has been shown to blunt the acute cellular and whole body hypoxic responses).

Thus, chronic hypoxia appears to be a central theme in adaptive responses during both physiological and pathological processes. Since maxiK activity is expressed in many O₂-sensing tissues and maxiK activity is acutely regulated by hypoxia, we proposed that one method of long term modulation by chronic hypoxia might involve functional remodeling of the maxiK channel complex itself. To test this hypothesis, we have studied the effect of long term, severe hypoxia (pO₂ ~18 mm Hg for 3 days) on channel expression and function, employing HEK293 cells stably co-expressing human α/β-maxiK subunits. The notion that chronic hypoxia exerts a dramatic influence on maxiK function is demonstrated by electrophysiological and biochemical techniques which show increased current density, altered Ca²⁺ sensitivity of acute O₂ regulation, dynamic and differential up-regulation of the β-subunit protein, and augmented co-localization of the α- and β-subunits in the plasma membrane.

MATERIALS AND METHODS

Cell Culture—HEK293 cells, which express human brain αβ-maxiK channels (40), were maintained in Earle’s minimal essential medium (containing L-glutamine) supplemented with 10% fetal calf serum, 1% antibiotic antimycotic, 1% non-essential amino acids, and 0.2% gentamicin (all purchased from Invitrogen) in a humidified incubator under 5% CO₂, 95% air. Cells were passaged every 3 days in a ratio of 1:2 using Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS, Invitrogen). The co-expressed α- and β-subunits were KCNMA1 (GenBank™ accession no. NM_002247) and KCNM1 (GenBank™ accession no. U61536), respectively. HEK293 cells expressing the same α-subunit but in the absence of the β-subunit were also used for one set of experiments. For the experiments described herein, cells were cultured for up to 3 days at 37 °C in water-saturated environments of either 2.5% O₂, 5% CO₂, 92.5% N₂ (pO₂ ~18 mm Hg, chronic hypoxia) or 21% O₂, 5% CO₂, 74% N₂ (pO₂ ~143 mm Hg, normoxia).

Electrophysiology—Unless stated otherwise, all chemicals for whole cell patch-clamp recordings were of the highest grade available and were purchased from Sigma Chemical Company. Pipette solution was K⁺-rich and contained (in mM): 10 NaCl, 117 KCl, 2 MgSO₄, 10 HEPES (pH 7.2 with KOH), 2 Na₂ATP with Ca²⁺-buffered to 4 mM, 27 Na₇, 300 mM, and 3 μM using EGTA and CaCl₂ in appropriate ratios. Bath solution was Na⁺-rich and contained (in mM): 135 NaCl, 5 KCl, 1.2 MgCl₂·6 HEPES, 2.5 CaCl₂, 10 γ-glucose (pH 7.4 with NaOH). All tubing was gas-impermeant (Tygon tubing, BDH). For the experiments in which pO₂ was changed acutely, normoxic solutions for a five-minute period were used with medical air while solutions were made hypoxic by bubbling with N₂ (pO₂) for at least 20 min. prior to perfusion of cells. This procedure produced no shift in bath pH or temperature. Solution flow rate was ~7–5 ml/min. pO₂ was measured close to the cell using a polarized pO₂ probe (SiM-100M, World Precision Instruments, Inc.). All solutions were equilibrated with 5% CO₂, 95% air before and were bubbled with a mixture of 5% CO₂, 95% air during the first 20 ms (Iend/Imax). Statistical comparisons were made using paired or unpaired Student’s t tests, as appropriate, with p < 0.05 being considered significant.

Immunoblotting—The expression of maxiK α- and β-subunits was measured by immunoblotting of crude cell extracts taken from confluent cell layers as follows. The confluent cell layer was washed twice with PBS without Ca²⁺ and Mg²⁺ and removed by scraping into SDS-PAGE sample buffer without 2-mercaptoethanol and bromphenol blue (1 ml per 75 cm² flask). The cell lysate was transferred to an Eppendorf microcentrifuge tube and heated in a boiling water bath for 10 min. A sample (100 μl) was taken for protein assay and replaced with 100 μl of 2-mercaptoethanol and 10 μl of a saturated solution of bromphenol blue. The cell extract was boiled for a further 10 min and equal protein amounts were analyzed by SDS-PAGE to allow direct comparison between samples. With this procedure the antibody was pre-adsorbed with the sequence (which has only one residue mismatch with the corresponding sequence in human β1-subunit) using 2 μg of peptide per 1 μl of undiluted antibody for 1 h at room temperature. After thorough washing of membranes, immunoblabeled proteins were detected using enhanced chemiluminescence (ECL, Amersham Biosciences), according to the manufacturer’s instructions. The level of maxiK subunit expression in the chronic hypoxic cells was expressed as a percentage of that found in the normoxic cells by densitometric analysis using the Quantity One densitometry package (Bio-Rad, Hemel Hempstead, Herts., UK).

Immunocytochemistry—Cells were grown on glass coverslips for 72 h under normoxic or chronic hypoxic conditions. The cells were washed twice with PBS containing 1 mM CaCl₂ and then fixed for 5 min with 10% formalin. The cell monolayer was rinsed twice with PBS containing 1 mM sodium azide and 5% normal goat serum (blocking solution) for 3 h to block nonspecific binding. The cells were then incubated overnight in 1:100 dilution of rabbit anti-maxiK antibody (from Transduction Laboratories, Lexington, KY) diluted 1:250 in crude anti-sodium and potassium channel rabbit antiserum residues 58–75 of rat BK β1-subunit (Biochem Pharm, Innsbruck, Austria) at a dilution of 1:6000. In some experiments the antibody was pre-adsorbed with the sequence (which has only one residue mismatch with the corresponding sequence in human β1-subunit) using 2 μg of peptide per 1 μl of undiluted antibody for 1 h at room temperature. After thorough washing of membranes, immunolabeled proteins were detected using enhanced chemiluminescence (ECL, Amersham Biosciences), according to the manufacturer’s instructions. The level of maxiK subunit expression in the chronic hypoxic cells was expressed as a percentage of that found in the normoxic cells by densitometric analysis using the Quantity One densitometry package (Bio-Rad, Hemel Hempstead, Herts., UK).

Nuclear Run-on Transcription Assay—Isolation of Nuclei: HEK293 cells expressing human αβ-maxiK channels were cultured in 150-cm² flasks for 2–1 h. Cells were processed in the same way, except that the primary antibodies were omitted from the initial incubations. This resulted in a lack of specific fluorescent staining in all cases (not shown).

Resistive feedback voltage-clamp was achieved using an Axopatch 200B amplifier (Axon Instruments, Forster City, CA). Voltage protocols were generated and currents recorded using pClamp 8 software employing a Digidata 1310 A/D converter (Axon Instruments). Data were filtered (4-pole Bessel) at 1 kHz and digitized at 5 kHz. Following successful transition to the whole cell recording a guide (2) containing transients were compensated and measured. To evoke ionic currents in HEK293 cells, three voltage protocols (V₀ = −70 mV, 0.1 Hz) were used: (1) a voltage ramp protocol, −100 mV to +60 mV, ramp duration = 1 s; 2) a time series, single increment to +60 mV, step duration = 50 ms; 3) single inactivating step protocol to +100 mV for 200 ms. The magnitude and steady-state currents were then divided by the final 5 ms of the +60 mV step and current density-voltage relationships were constructed from the voltage ramp data. Inactivation data were taken from the 200-ms step; peak current was the maximum achieved during first 20 ms, and final current was measured during the final 5 ms of the step. Inactivation was expressed as proportion of current still available at end of step as proportion of the maximum measured during the first 20 ms. Statistical comparisons were made using paired or unpaired Student’s t tests, as appropriate, with p < 0.05 being considered significant.

After thorough washing of membranes, immunolabeled proteins were detected using enhanced chemiluminescence (ECL, Amersham Biosciences), according to the manufacturer’s instructions. The level of maxiK subunit expression in the chronic hypoxic cells was expressed as a percentage of that found in the normoxic cells by densitometric analysis using the Quantity One densitometry package (Bio-Rad, Hemel Hempstead, Herts., UK).
phenylmethylsulfonyl fluoride, 1 mM spermidine, 1 mM diethiothreitol, and 0.1% (v/v) Triton X-100 and then sonicated (20 strokes). An equivalent volume of sucrose buffer II was added to the nuclei preparation (2.1 st sucrose, 5 mM MgAc2, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8, 1 mM EGTA containing 1 mM spermidine, 1 mM diethiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) followed by gentle vortexing. This mixture was layered on top of a 4 ml sucrose cushion (sucrose buffer II) and nuclei were purified by centrifugation for 50 min at 16 000 x g at 4 °C. Purified nuclei were re-suspended in 200 μl of storage buffer (40% v/v) glycerol, 10 mM Tris-HCl, pH 8, 5 mM MgCl2, 0.1 mM EDTA, 1 mM EGTA containing 0.1 mM phenylmethylsulfonyl fluoride and 1 mM spermidine) and frozen at -80 °C until required. Nuclei concentration was determined by counting the number of viable nuclei present in a 1:100 dilution of each sample using an electronic counter (Qiagen), and ligated into pGEM-T Easy cloning vector (Promega). Constructs were sequenced (Lark Technologies Inc, Saffron Walden, Essex, UK) to determine orientation and correct sequence of inserts. Specific primers for the T7 or SP6 promoter sequences were used in conjunction with internal primers specific for either α-subunit, β-subunit, or β-actin, to generate PCR products from the pGEM-T Easy which would act as templates for antisense RNA probe production. Primer pairs used were as follows: SP6pGEM 5'-ATTTAGGTGACAC-TATAGAA-3' and BkNpNA 5'-ACCCGGCGCTATGAGTTG-3' for maxiK α probe template; SP6pGEM and BKnpNA 5'-GGCCCTCCTC-TCTCCTCTTTT-3' for maxiK β probe template; T7pGEM 5'-TAATAC-GACGACTCACTATAGGG-3' and ActNAc 5'-CAACCGCGAGAAGTACCGACAGTCCATGCTGAAAGGT-3' for β-actin probe. Probe template DNA was purified on QIAquick PCR purification columns. Radioabeled antisense RNA probes were synthesized using the MAXIScript in vitro transcription kit (Ambion, Huntingdon, Cambs., UK). In brief, 0.2 μg of template DNA was added to a reaction mixture containing 1× transcription buffer, 0.5 μM ATP, 0.5 μM CTP, 0.5 μM GTP, 1 μM TTP (50 Ci mmol⁻¹) and either 40 units of SP6 or 30 units of T7 enzyme mix in a final volume of 20 μl. In addition to the above components, 0.5 μM unlabeled UTP was added to the β-actin probe template to reduce the specific activity of this RNA probe. All reactions were incubated at 37 °C for 10 min and template DNA was removed by the addition of 2 units of RNase I and a further 10 min incubation at 37 °C. RNA probes were purified by electrophoresis through a 4% acrylamide/8 % urea gel following which the appropriate bands were excised and the probe eluted by incubating at 37 °C overnight in nuclease-free water.

**Nuclease Protection Assay—**Total RNA for nuclease protection assays was isolated from cells following 3 days incubation under either chronic hypoxic or normoxic conditions. RNA was isolated using TRIzol reagent following the manufacturer’s protocol (Invitrogen). All assays used the ribonuclease protection assay kit (RPA III) purchased from Ambion. In brief, either 5, 15, or 25 μg of total RNA was mixed with 8 x 10⁴ cpm β-actin probe, 8 x 10⁴ cpm maxiK α probe, and 2 x 10⁴ cpm maxiK β probe. Two yeast RNA samples were also prepared as controls. P32-labeled RNA were then co-precipitated with 1 μg ActNAc and 5 μg poly (dI) · (dC) and 5 μg poly (dI) · (dC) and were then dissolved in 5 μl of water and incubated at –20 °C for at least 15 min, followed by centrifugation at 16,000 x g for 15 min at 4 °C. Supernatants were aspirated and pellets resuspended in 10 μl of RPAIII hybridization buffer. Samples were heated at 95 °C for 4 min followed by an overnight incubation at 42 °C. A 1:100 working dilution of RNase A/RNase T1 in RNase digestion III buffer was prepared and 150 μl was added to each of the sample tubes and one of the yeast tubes, no RNase was added to the second yeast tube. All the reactions were incubated for 30 min at 37 °C before being terminated by the addition of 225 μl of RNase inactivation/precipitation solution. The samples were incubated using a vortexer for at least 20 min followed by centrifugation at 16,000 x g for 15 min at 4 °C. The supernatant was carefully removed without disturbing the RNA pellets, and RNA was dissolved in 10 μl of gel loading buffer. Samples were incubated for 3 min at 95 °C and placed briefly on ice before being loaded onto a 4% acrylamide/8 M urea gel electrolysed at 250 volts until the bromophenol dye band reached the bottom of the gel. The gel was then exposed to x-ray film for several days before developing.

**RESULTS**

**Chrono Hypoxic Augments maxiK Current Density—**Fig. 1, A-D shows exemplar currents elicited in voltage-clamped HEK293 cells stably co-expressing recombinant human maxiK channels during the voltage-ramp protocol following incubation in normoxia (pO₂ - 143 mm Hg; 72 h) or chronic hypoxia (pO₂ - 18 mm Hg; 72 h). Experiments were carried out in the presence of [Ca²⁺]₁, buffered to the levels indicated in each panel. At
FIG. 1. Chronic hypoxia enhances the current density of recombinant maxiK channels stably co-expressed in HEK293 cells. Current density-voltage relationships derived from the voltage ramp protocol recorded from exemplar normoxic and chronically hypoxic cells with 

\[ \text{[Ca}^{2+}] \] buffered to 4 nM (A), 27 nM (B), 300 nM (C), and 3 μM (D). E, mean (±S.E.) effects of [Ca\(^{2+}\)] on recombinant maxiK current densities in normoxia (closed circles, n = 5–10) and chronic hypoxia (open symbols, n = 5–9). * and ** denote statistically significant differences between normoxic and chronic hypoxic cohorts at \( p < 0.05 \) and \( p < 0.005 \), respectively (Student's unpaired t test). For comparison, untransfected HEK293 cell current densities at 300 nM Ca\(^{2+}\), are plotted as closed (normoxia) and open (chronic hypoxia) squares. Note that the chronic hypoxic symbol has been offset to aid clarity. Inset shows currents recorded from normoxic and hypoxic cells during the step depolarization indicated below the current traces.
all levels, chronic hypoxia caused an increase in current density. Chronic hypoxia had no effect on cell capacitance with the normoxic cell cohort demonstrating a mean capacitance of 10.7 ± 0.5 pF (n = 32) and the chronic hypoxic cell cohort demonstrating a mean capacitance of 10.4 ± 0.6 pF (n = 27). The mean increase in current density are quantified in Fig. 1E, which compares the [Ca\(^{2+}\)\] dependence of αβ-maxiK currents (measured from the +60 mV step) cultured in either normoxia or chronic hypoxia. As expected for these K\(^+\) channels, current flowing through this conductive pathway was activated in a graded manner as [Ca\(^{2+}\)] was increased; an effect which was seen in cells cultured in either normoxia or hypoxia. Over the physiologically important [Ca\(^{2+}\)] range (27–300 nM), chronic hypoxia evoked a large and significant augmentation in current density, whereas at the lowest and highest levels studied, current densities were not significantly altered (Fig. 1E). Thus, the [Ca\(^{2+}\)] window that supported hypoxic stimulation was essentially within the physiological range. The time course of this regulatory effect was relatively slow with notable up-regulation by chronic hypoxia not apparent until at least 24 h into the experimental exposure. In this particular series of experiments ([Ca\(^{2+}\)] = 27 nM); 24 and 48 h of chronic hypoxia did not evoke significant (p > 0.5) changes in current density (55.0 ± 6.1 pA pF\(^{-1}\); n = 10), 64.6 ± 17.8 (24 h; n = 4), 50.5 ± 13.12 pA pF\(^{-1}\); (n = 5). However, 72 h exposure to chronic hypoxia caused a highly significant augmentation to 117.6 ± 18.5 pA pF\(^{-1}\) (n = 7).

In untransfected HEK293 cells, even at [Ca\(^{2+}\)] of 300 nM, current density was low (10.8 ± 3.2 pA pF\(^{-1}\); n = 6) and this was not significantly altered following 72 h in chronic hypoxia (8.6 ± 0.9 pA pF\(^{-1}\); n = 6). These values compare with transfected cells which demonstrated current densities of 137.1 ± 30.4 pA pF\(^{-1}\) (n = 9, normoxia) and 285.0 ± 68.9 pA pF\(^{-1}\) (n = 6, chronic hypoxia) at the same level of [Ca\(^{2+}\)].

Thus, native HEK cell currents represented ~8% of normoxic current density and ~3% of chronic hypoxia-induced current (Fig. 1E). Furthermore, cell attached recordings (measured just prior to achieving whole cell configuration) demonstrated single channels with a mean conductance of 151.0 ± 10.1 pS (Vp = +120 mV, K\(^+\) pipette, n = 5) showing the presence of maxiK channels, an observation, which is consistent with our previous studies in this cell line (27). Such single channel events were never seen in native HEK293 cells (data not shown, n = 14).

Inactivation was studied during a 200-ms pulse from −70 mV to +100 mV (Fig. 1E, inset). Currents recorded from normoxic cells demonstrated no inactivation during this test pulse (I\(_{\text{out}}\)I\(_{\text{max}}\) = 0.97 ± 0.04, n = 5). In complete contrast, cells cultured for 72 h in chronic hypoxia showed significant inactivation during depolarizing steps (I\(_{\text{out}}\)I\(_{\text{max}}\) = 0.75 ± 0.04, n = 4). Inactivation during this 200-ms pulse was significantly different in the two cohorts of cells (p < 0.01).

**Chronic Hypoxia Modulates the Ca\(^{2+}\) Dependence of Acute O2 Sensing Only When the β-Subunit Is Present—**Fig. 2A shows exemplar K\(^+\) currents elicited before and during acute reduction in bath pO\(_2\) from 150 mm Hg to ~25 mm Hg with [Ca\(^{2+}\)], buffered to 27 nM. The mean time-series plot (Fig. 2B) illustrates the time course of this effect of acute hypoxia and highlights the fact that acute hypoxia was only able to inhibit currents at this [Ca\(^{2+}\)], in cells maintained in chronic hypoxia.

Consistent with our previous study which employed excised patches from this cell line (27), the recombinant human αβ-maxiK whole cell current recorded from cells cultured in normoxia showed a [Ca\(^{2+}\)]-dependent sensitivity to acute hypoxia (Fig. 2C). Although this effect of acute hypoxia on whole currents was qualitatively similar to that demonstrated in inside-out patches, the sensitivity of the system was shifted toward lower, more physiologically pertinent [Ca\(^{2+}\)], i.e. whole cell currents were inhibited maximally by hypoxia at 300 nM [Ca\(^{2+}\)], (see Fig. 2C) whereas macro currents from inside-out patches were inhibited maximally by hypoxia at [Ca\(^{2+}\)], greater than 1 μM (see Fig. 4 in Ref. 27). Thus, in normoxic cells with low [Ca\(^{2+}\)], the maxiK whole cell current demonstrated little or no acute hypoxic sensitivity while acute hypoxic sensitivity was greatly increased at the higher [Ca\(^{2+}\)], (Fig. 2C). Chronic hypoxia significantly changed this pattern of Ca\(^{2+}\) dependence of the acute hypoxic response (Fig. 2C) such that a substantial inhibition of K\(^+\) current in response to acute hypoxia was observed even at low [Ca\(^{2+}\)]. At higher [Ca\(^{2+}\)], the cells cultured in chronic hypoxia demonstrated a mildly decreased sensitivity to acute hypoxia and there were no significant differences between acute hypoxic inhibition between the normoxic and chronic hypoxic cells. Therefore, chronic hypoxia significantly increased the Ca\(^{2+}\) dependence of the acute hypoxic response such that channel inhibition was enabled even at very low [Ca\(^{2+}\)].

In contrast to the results from cells co-expressing the α- and β-subunits, chronic hypoxia completely failed to increase current density or modulate acute hypoxic sensitivity in a separate HEK293 cell line stably expressing only the α-subunit of the maxiK channel complex. Thus, mean current density, at [Ca\(^{2+}\)], of 27 nM, was 98.7 ± 15.2 pA pF\(^{-1}\) following 72 h in normoxia and 94.2 ± 12.3 pA pF\(^{-1}\) following 72 h in chronic hypoxia (n = 5 for both).

**Chronic Hypoxia Evokes a Selective Up-regulation of the β-Subunit—**In order to investigate the molecular nature of the functional change in acute hypoxic sensitivity evoked by chronic hypoxia, we studied the effect of chronic hypoxia on maxiK α- and β-subunit expression levels using Western blotting and immunocytochemistry. Fig. 3 shows Western blots of total cell lysates from cells cultured under normoxic or chronic hypoxic conditions. Using an antibody which was specific for the α-subunit, a major band of the expected size was detected (125 kDa, Fig. 3A, left panel) and there was no significant difference between the mean optical densities of this band in either condition (Fig. 3B, n = 5). When antisera to the β-subunit was used, two bands were detected (Fig. 3A, center panel). The higher molecular weight band was of ~35 kDa, consistent with a previous report of heavy glycosylation of this protein, which always appears as a band of molecular weight in excess of 30 kDa on SDS-PAGE gels (46). The notion that this upper band represented immunoreactivity to the β-subunit was supported by the data in Fig. 3B (right panel) which shows the complete removal of this band following pre-absorption with the peptide against which the antisera was raised; the lower band was not removed by this pre-absorption step and, therefore, represents immunoreactivity of an unrelated peptide. The mean optical density of the β-subunit band was significantly increased in the cells cultured in chronic hypoxia (Fig. 3B, n = 6).

Selective increase in β-subunit protein in response to chronic hypoxia was confirmed by confocal immunocytochemistry using antibodies selective for the α- and β-subunits of maxiK (Fig. 3C). In the normoxic cells, the α-subunit staining (green) was localized mainly at the periphery of the cells, consistent with plasma membrane location (Fig. 3Ca). Some intracellular staining was also detected presumably due to ongoing protein trafficking. In the cells cultured in chronic hypoxia, there was no noticeable change in either intensity or localization of α-subunit staining (Fig. 3Cd). On the other hand, β-subunit staining (red) was diffuse in normoxia (Fig. 3Cb), but was clearly more intense following chronic hypoxia (Fig. 3Ce). This observation was consistent with the up-regulation of β-subunit expression.
FIG. 2. Chronic hypoxia modulates the Ca\(^{2+}\) sensitivity of the acute hypoxic inhibition of recombinant maxiK channels. A, exemplar current density-voltage relationships derived from the voltage ramp protocol recorded before and during acute reduction of pO\(_2\) from \(~150\) mm Hg (Acute normoxia) to 15–25 mm Hg (Acute hypoxia) in exemplar cells, which had been cultured for 3 days in either normoxia (Normoxia) or chronic hypoxic (Chronic hypoxia) with [Ca\(^{2+}\)]\(_i\) buffered to 27 nM. B, mean (±S.E.) time series plots of K\(^+\) current amplitudes recorded at 60 mV demonstrating the effect of chronic hypoxic modulation on the acute hypoxic response of maxiK\(^+\) channels. Time series from normoxic cells are shown using open symbols (\(n = 9\)) and those from chronic hypoxic cells using closed symbols (\(n = 5\)). Application of hypoxic perfusate is indicated by horizontal bar. C, mean (±S.E.) effects of [Ca\(^{2+}\)]\(_i\) on the acute hypoxic sensitivity of recombinant maxiK\(^+\) in normoxic (open symbols, \(n = 5–14\)) and chronic hypoxia (closed symbols, \(n = 5–9\)). * denotes statistically significant differences between normoxic and chronic hypoxic cohorts at \(p < 0.05\) (Student’s unpaired t test).
shown by Western blotting (Fig. 3, A and B) and functional data shown in Figs. 1 and 2. Furthermore, the α- and β-subunit immunoreactivity overlays suggest a possible increased co-localization of the subunits (yellow staining) in response to chronic hypoxia (compare Fig. 3, Cc to Cf).

**Chronic Hypoxic Up-regulation of the β-Subunit Is a Post-transcriptional Event**—To determine whether hypoxia caused an increase in β-subunit gene transcription, we employed the novel approach of nuclear run-on followed by real-time PCR to measure transcription rate. Melt curve analyses showed the presence of single peaks at 85 °C and 89 °C for α- and β-subunit RT-PCR products, respectively (data not shown), indicating specificity of the PCR primer pairs. Agarose gel electrophoresis confirmed this result, showing single products of the desired length (α-subunit = 206 bp, β-subunit = 189 bp) with no bands observed in the negative control lanes (Fig. 4A, inset), indicating very little primer dimer structure. Fig. 4A shows an example of a real-time PCR experiment performed on a dilution series of template cDNA using α-subunit primers. This information and data obtained from sim-
ilar experiments performed with the β-subunit primer pairs (data not shown), was used to produce standard curves generated from the crossing point parameter of the cDNA dilution series (Fig. 4B). Good correlation was found between log-copy numbers and crossing points with regression coefficient values of over 0.99. The slopes of the standard curves were used to determine the PCR efficiencies of α- and β-subunits as 1.90 and 2.15, respectively, which are within the acceptable range for LightCycler real-time PCR.

Run-on experiments were performed for both α- and β-subunits on nuclei isolated from normoxic and chronic hypoxic cells. Fig. 4C shows a typical result; addition of NTPs caused a shift in crossing point cycle number to a lower value, indicating the presence of more cDNA compared with the minus NTP samples. This was due to transcription occurring within the 30-min incubation period of the nuclear run-on reaction. Comparison of the amounts of nascent mRNA obtained following normoxia or chronic hypoxia showed no significant difference for either subunit (Fig. 4D). The mean (S.E.) ratios of nascent mRNA formed in the normoxic and chronic hypoxic samples were 1.018 ± 0.071 and 0.962 ± 0.161 for the α- and β-subunits, respectively, indicating that chronic hypoxia did not significantly alter transcription rates of either subunit.

Steady state levels of mRNA of α-subunit, β-subunit, and β-actin were determined using a nuclease protection assay and Fig. 5A shows a typical gel autoradiograph. Full-length probes were detected in the sample containing yeast RNA without RNase (lane Y−; β-actin 536 bp; maxiKα 268 bp and maxiKβ 312 bp). Little nonspecific binding of probes was detected in the yeast sample with RNase (lane Y+). Protected products of the predicted sizes were observed in all experimental samples (β-actin 470 bp; maxiKα 185 bp; and maxiKβ 230 bp). The intensity of the bands corresponding to the protected products of α- and β-subunits were normalized to that of the β-actin band within each lane, allowing comparison of the maxiKα- and β-subunit mRNA levels under normoxic and chronic hypoxic conditions. No significant differences in mRNA steady-state levels were observed for either of the subunits. This finding was verified by scintillation counting quantification.
Together, the data presented in Figs. 4 and 5 indicate that chronic hypoxia alters neither transcription rate nor mRNA stability of the maxiK subunits.

**DISCUSSION**

**Potential Molecular Mechanism of Chronic Hypoxic Regulation**—Through the use of a mammalian recombinant expression system, our whole cell patch-clamp data have demonstrated that acute hypoxia inhibits maxiK channels of known molecular identity in a manner qualitatively similar to that previously observed in excised inside-out patches (27). However, in the whole cell configuration, the Ca$^{2+}$/H$^{+}$ dependence of the acute hypoxic response is shifted into the normal physiological [Ca$^{2+}$] range. This patch-clamp configuration has also demonstrated that chronic hypoxia results in a significant up-regulation of K$^{+}$ current density and even further increases in the Ca$^{2+}$/H$^{+}$ sensitivity of the acute hypoxic response. Furthermore, this work has revealed important new findings that suggest strongly that chronic hypoxic remodeling of maxiK function is due to differential increase in β-subunit protein that is possibly associated with enhanced co-localization of the β-subunit with the α-subunit at the plasma membrane.

That chronic hypoxia evokes increased functional α/β multimers at the plasma membrane is fully compatible with the role of the β-subunit in channel Ca$^{2+}$/H$^{+}$ sensitivity. Thus, a higher proportion of the functional channels would be predicted to contain the β-subunit in their protein complex, which would produce a larger population of channels with increased Ca$^{2+}$ sensitivity. As a consequence, channels are able to respond to acute hypoxia at lower [Ca$^{2+}$]. In addition, chronic hypoxia results in an increased rate of inactivation seen during step depolarizations. This observation is wholly compatible with augmented number of α/β heteromers contributing to the K$^{+}$ current under these conditions since it has been recently reported that inactivation of maxiK channels increases as the transfection ratio of β-subunit:α-subunit is steadily increased (47). Further compelling evidence that functional up-regulation by chronic hypoxia is dependent upon remodeling of the channel subunit ratio at the plasma membrane is our direct observation that no such up-regulation occurs in cells expressing only the α-subunit.
The nuclear run-on assays demonstrate clearly that transcriptional control is unimportant to long term regulation of maxiK channel function in this recombinant model. Furthermore, maxiK mRNA half-life is not altered by chronic changes in $O_2$ since the RNase protection assays indicate no change in steady-state mRNA levels. Use of this stable cell line has, therefore, allowed us to investigate directly the involvement of post-transcriptional events in chronic hypoxic up-regulation of maxiK channel function without the confounding variables of transcriptional control or mRNA stability. Of course, in cells natively expressing maxiK channels, there may well be additional regulation at the level of the synthesis and degradation of maxiK gene products, a situation that has been previously suggested for chronic hypoxic regulation of tyrosine hydroxylase in PC-12 cells (48). However, when the impact of such putative regulatory mechanisms is removed, we have been able to reveal that chronic hypoxia, via a mechanism that must be located downstream of mRNA regulation, is able to alter differentially maxiK channel $\beta$-subunit protein abundance, localization and, as a consequence, function. Indeed, that the up-regulation reaches significance only by 72 h demonstrates clearly that the effect cannot be explained by rapid regulatory processes (via, for example, phosphorylation or insertion of pre-assembled channels) but is due to long-term remodeling. Such an assumption is clearly compatible with post-transcriptional regulation and there is evidence in the literature that changes in $[Ca^{2+}]$ cannot only affect transcription rates of certain genes but also the rates of protein synthesis (see, for example, Ref. 49). Further, hypoxia-evoked regulation of protein synthesis has been previously described and likely occurs via $[Ca^{2+}]$-induced phosphorylation of initiation factor 2 (eIF2) (50, 51), a mechanism that may be similar to that which we have described herein for maxiK channel $\beta$-subunits. 

Potential Role for Regulation of Channel Function by Chronic Hypoxia—MaxiK channels are widely distributed and, as such, are of importance to many physiological processes (1). For example, they are intimately involved in control of neuronal excitability (14), chemosensing (52, 53) and, controversially, control of vascular tone (54, 55). In chemosensory tissues, episodes of acute hypoxia result in modulation of ventilation and optimization of ventilation-perfusion matching via the concerted actions of the carotid bodies, the neuroepithelial bodies of the lung and the pulmonary vasculature (1, 6, 11, 15). Evidence supports the idea that hypoxic inhibition of maxiK channels is central to some of these effects. There is clear evidence for divergent mechanisms underlying the initial steps in $O_2$ signal transduction in different chemosensory tissues (for recent review see Ref. 11) although the direct modulation of the channel complex, or closely associated protein partners, by $O_2$ is clearly the most favored mechanism for native neuronal (17, 56) and recombinant neuronal (27) maxiK channels. Assuming that direct regulation is one of the most likely modes, it follows that any factor that can modulate subunit transcription, translation, trafficking, assembly, or a combination of these processes, will provide a powerful stimulus for functional maxiK channel remodeling. It is clear from the data presented herein and elsewhere, that ambient $O_2$ represents such a remodeling cue. Using our recombinant model we have been able to "uncouple" transcriptional and post-transcriptional control by chronic hypoxia and, thereby, have the direct evidence that chronic hypoxia selectively alters the synthesis, retrieval or insertion of an identified ion channel subunit. This is a particularly exciting observation when one considers how widespread the phenomena of generalized or localized hypoxia are in pathophysiological conditions. For example, one paradigm of intermittent chronic hypoxia that affects almost a quarter of the adult male population, obstructive sleep apnea, is strongly associated with hypertension and predisposition to cerebral vascular accident or stroke (32). It is tempting to speculate that one underlying reason for the increased susceptibility of these patients to stroke may involve functional remodeling of neuronal maxiK channels by this chronic stimulus such that they are much more sensitive to $O_2$ at low resting $[Ca^{2+}]$ (as shown in Fig. 2). If that is the case, when cells are subjected to acute focal hypoxia/ischemia (as occurs during stroke), inhibition of these remodelled $K^+$ channels would be predicted to cause an augmented depolarizing response and concomitant exacerbation of neuroexcitotoxicity.

The evidence available suggests that expression of $\alpha$-subunit protein at the plasma membrane in cells cultured in normoxia is far higher than that of the $\beta$-subunit. Such a suggestion is based upon the relatively low $Ca^{2+}$ sensitivity, which we see (in inside-out patches and whole cell recordings) coupled to the rather diffuse immunohistochemical staining observed with the $\beta$-subunit and the lack of inactivation. Interestingly, $\beta$-subunit expression in many brain areas is also much less than in smooth muscle cells, for example (57), suggesting that the cells which we employ here might be a suitable model for the study of potential alterations in subunit stoichiometry that occurs during chronic perturbations in $pO_2$.

In conclusion, we have shown the function of identified, human maxiK channels is dramatically remodeled by pathologically pertinent chronic reductions in environmental $pO_2$. This remodeling may underlie some of the numerous pathological conditions, which are characterized by chronic and intermittent hypoxia such as chronic obstructive pulmonary disease or congestive heart failure.

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