# A cysteine-rich motif confers hypoxia sensitivity to mammalian large conductance voltage- and Ca-activated K (BK) channel $\alpha$-subunits

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**Cellular responses to hypoxia are tissue-specific and dynamic. However, the mechanisms that underlie this differential sensitivity to hypoxia are unknown. Large conductance voltage- and Ca-activated K (BK) channels are important mediators of hypoxia responses in many systems. Although BK channels are ubiquitously expressed, alternative pre-mRNA splicing of the single gene encoding their pore-forming $\alpha$-subunits provides a powerful mechanism for generating functional diversity. Here, we demonstrate that the hypoxia sensitivity of BK channel $\alpha$-subunits is splice-variant-specific. Sensitivity to hypoxia is conferred by a highly conserved motif within an alternatively spliced cysteine-rich insert, the stress-regulated exon (STREX), within the intracellular C terminus of the channel. Hypoxic inhibition of the STREX variant is Ca-sensitive and reversible, and it rapidly follows the change in oxygen tension by means of a mechanism that is independent of redox or CO regulation. Hypoxia sensitivity was abolished by mutation of the serine (S24) residue within the STREX insert. Because STREX splice-variant expression is tissue-specific and dynamically controlled, alternative splicing of BK channels provides a mechanism to control the plasticity of cellular responses to hypoxia.**

alternative splicing | KCNMA1 | oxygen sensing

Mammalian cell survival depends on the presence of oxygen. The lowering of oxygen tension (hypoxia) (whether from the disruption of blood flow, inhibition of gaseous exchange, or changes in cellular metabolism) can trigger a range of physiological responses that attempt to minimize the detrimental effects of hypoxia. Large-conductance Ca- and voltage-activated K (BK) channels have been identified as one of the key mediators of the response of the body to hypoxia. BK channels are important for the "oxygen-sensing" function of specialized tissues, such as the carotid body and neuroepithelia (1–3), as well as for determining cellular excitability in smooth muscle and neurons (4, 5). However, the responsiveness of native BK channels to changes in oxygen tension is as diverse as the tissues in which they are expressed, with some being completely insensitive to hypoxia (6) and others being potently inhibited by hypoxia (1–3). Also, cellular and tissue sensitivity to hypoxia are highly plastic (7–9), with adaptive responses that depend on prior and prevailing conditions, which may involve changes in BK channel expression (10), although the underlying mechanisms are essentially unknown.

The pore-forming $\alpha$-subunits of BK channels are encoded by a single gene (11), *KCNMA1*, which undergoes extensive alternative pre-mRNA splicing (12, 13). The $\alpha$-subunits assemble as tetramers to form functional channels (14, 15). Distinct splice-variant mRNAs of $\alpha$-subunits may be expressed in the same cell or differentially expressed between tissues or even neighboring cells (16, 17). Dynamic modification of splice-variant mRNA expression (18, 19) allows plasticity in BK channel phenotype and cellular regulation (20–22). Functional diversity can also be generated by coassembly of $\alpha$-subunits with a family of regulatory transmembrane $\beta$-subunits (23). Expression of distinct $\beta$-subunits may also be tissue-specific; for example, $\beta_1$-subunits are largely restricted to cells of the vasculature, whereas $\beta_4$-subunits are highly expressed in the nervous system (24–26).

Coexpression of BK channel $\alpha$-subunits with the $\beta_1$-subunit in human embryonic kidney (HEK) 293 cells resulted in BK channels that were potently inhibited by hypoxia by a heme oxygenase (HO2)- and CO-dependent mechanism (2). It is not known whether $\alpha$-subunits of BK channels alone are hypoxia sensitive, nor is it known whether alternative splicing can switch the sensitivity of $\alpha$-subunits to changes in oxygen tension. Also, because $\beta_1$-subunit expression is largely restricted to vascular smooth muscle cells and $\beta_1$-subunits are not expressed in several cell types in which native BK channels are reported to be sensitive to hypoxia (4), $\beta_1$-subunit coassembly cannot be essential for sensitivity to hypoxia.

To address these fundamental issues, we analyzed the effect of hypoxia on native and recombinant murine BK channels. Our studies reveal that alternative pre-mRNA splicing of the pore-forming $\alpha$-subunit provides a mechanism to generate functional diversity in the response of BK channels to hypoxia and that an evolutionary conserved motif within the alternatively spliced stress-regulated exon (STREX) insert is essential for sensitivity of BK channel $\alpha$-subunits to changes in oxygen tension.

## Materials and Methods

**Cell Culture and Transfection.** AtT20 D16:16 and HEK 293 cells were maintained in DMEM (GIBCO/BRL) with 10% FBS, as described in refs. 21 and 27. HEK 293 cells grown on glass coverslips were transfected with the appropriate cDNA (1 $\mu$g/ml) by using Lipofectamine 2000 (Invitrogen), and they were used for electrophysiological recordings at 1–3 days after transfection, as described in ref. 21. To generate cells that stably expressed the transfected DNA, cells were selected by using the appropriate antibiotic.

**Construction of Expression Plasmids.** Construction of the murine ZERO, STREX, and IYF splice variants in the mammalian expression vectors pcDNA3 or 3.1 have been described (21, 27). The site-directed mutants STREX-C23A:C25A and STREX-S24A were generated by QuikChange site-directed mutagenesis

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Abbreviations: BK, large conductance voltage- and Ca-activated K channel; STREX, stress-regulated exon; HEK, human embryonic kidney; HO2, heme oxygenase 2; $V_{0.5max}$, half-maximal voltage for activation; $P_o$, open probability; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration.

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on a PacI–BlpI cassette, spanning site of splicing C2, according to the manufacturer's protocol (Stratagene). The mutated PacI–Blp1 cassette was subcloned into the PacI–BlpI sites of the full-length channel constructs. The following primer sequences were used to construct the STREX-C23A:C25A mutant: 3′-TCTGAGCGTGACGCCTCGGCCATGTCAGGC-5′ (sense) and 5′-GCCTGACATGGCCGAGGCGTCACGCTCAGA-3′ (antisense). The following primer sequences were used to construct the STREX-S24A mutant: 5′-TCTGAGCGTGACT-GCGCGTGCATGTCAGGC-3′ (sense) and 5′-GCCTGACAT-GCACGCGCAGTCACGCTCAGA-3′ (antisense). Mutations and sequence integrity were confirmed by the sequencing of both strands.

STREX-C23A:C25A channels had a slope conductance of 145 ± 5 pS ($n$ = 16) and Ca sensitivity [half-maximal voltage for activation ($V_{0.5max}$), 86 ± 2 mV and 62 ± 5 mV and in 0.1 and 1 μM intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), respectively; $n$ = 16] similar to those of the IYF variant ($V_{0.5max}$, 95 ± 4 mV and 48 ± 4 mV in 0.1 and 1 μM $[Ca^{2+}]_i$, respectively; $n$ = 12 and 16). For STREX-S24A, single-channel conductance was 135 ± 8 pS ($n$ = 8), with a $V_{0.5max}$ in 1 μM $[Ca^{2+}]_i$, of 42 ± 9 mV ($n$ = 4), which was between that of the wild-type STREX ($V_{0.5max}$ in 1 μM $[Ca^{2+}]_i$ of 32 ± 5 mV; $n$ = 16) and wild-type IYF.

**Electrophysiology.** Single-channel patch–clamp recordings of BK channels were made at room temperature (20–24°C). Patch pipettes were manufactured with borosilicate glass (Clark Electromedical Instruments, Pangbourne, U.K.) with resistances of 8–12 MΩ. Channel activity was recorded by using an Axopatch-1D or Axopatch 200A patch–clamp amplifier (Axon Instruments, Union City, CA). Single-channel data were recorded and analyzed by using either PCLAMP 9 (Axon Instruments) or WINEDR (Version 2.3.9; J. Dempster, University of Strathclyde). Channel activity was determined over the voltage range of −20 to +80 mV. In patches containing two or three channels, channel amplitude and open probability ($P_o$) were estimated from all-points amplitude histograms, which were determined from samples of 60 s in duration and fitted with Gaussian functions. In patches containing three to eight channels, $P_o$ was determined from the total current ($I$), the integral of the current recorded for 60 s, where $I = N_f{\cdot}P_o{\cdot}i$, with $N_f$ being the number of functional channels in the patch and $i$ being the single-channel current amplitude. To produce the best estimate of $P_o$, the maximum number of functional channels observed in a given patch was measured at the most depolarized potentials in the highest Ca concentration. Activation curves were fitted with a single Boltzmann function to determine the $V_{0.5max}$. Channel slope conductance was determined over the range of +20 to +60 mV, where the slope is approximately linear. The voltage sensitivity of the channels was calculated by the slope of a plot of $\ln[P_o/(1 - P_o)]$ against voltage. Mean channel $P_o$ at each time point was determined from samples of 60 s in duration.

**Solutions and Drugs.** Excised inside-out recordings were made in physiological K gradients by using a pipette solution (extracellular) containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM glucose, and 10 mM Hepes (pH 7.4). The bath solution (intracellular) contained 140 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, and 5 mM EGTA, or 1 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate (BAPTA), 30 mM glucose, and 10 mM Hepes (pH 7.3), with Ca added at the required concentration. The possibility that hypoxic inhibition was due to channel run down was excluded by performing experiments on isolated patches that were allowed to stabilize for ≥5–10 min after excision in the absence of ATP. Hypoxia had no effect on the pH or temperature of the bath solution. Inhibition was identical when $[Ca^{2+}]_i$ was buffered by either EGTA or BAPTA.

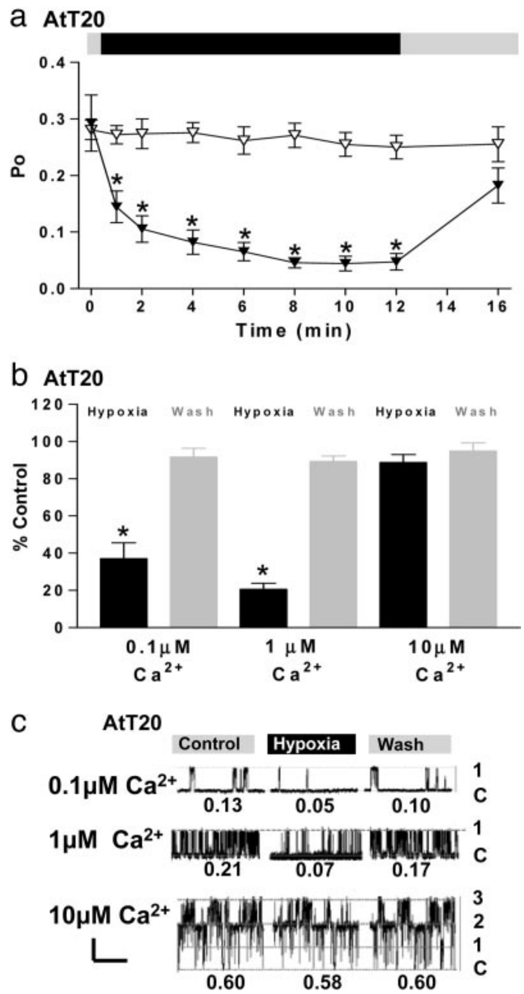


**Fig. 1.** Hypoxia inhibits the activity of endogenous BK channels in AtT20 corticotropes. (*a*) Hypoxia (black bar) significantly inhibited BK channel mean $P_o$ at 40 mV in 0.1 μM free Ca $[Ca^{2+}]_i$ (▼; $n$ = 12), compared with channel activity in normoxic saline (▽; $n$ = 12). (*b*) Hypoxic inhibition was reversible and Ca-dependent with significant inhibition of activity in 0.1 and 1 μM $[Ca^{2+}]_i$ but not in 10 μM $[Ca^{2+}]_i$ nor after wash ($n$ = 12). (*c*) Representative traces of single BK channel activity in AtT20 cells at 40 mV in 0.1 and 10 μM $[Ca^{2+}]_i$ and 20 mV at 1 μM $[Ca^{2+}]_i$. $P_o$ values are indicated for each trace. (Scale bar, 5 pA, 0.1 s; and 3 pA, 0.2 s; 1 μM $[Ca^{2+}]_i$ and 20 mV.) All data are given as mean ± SEM. *, $P < 0.01$, by ANOVA with post hoc test.

Solutions were added to the bath by using gravity-feed perfusion, and they were withdrawn by using an electric pump. Flow rates of 3–10 ml·min$^{-1}$ were used.

**Oxygen-Tension Measurement.** Hypoxia was achieved by bubbling solution with nitrogen gas for ≥20 min before use. The changes in oxygen tension ($pO_2$) of the bath during perfusion with hypoxic saline were recorded by using an oxygen microelectrode which had a response time of <20 s (model 781; StrathKelvin Instruments, Glasgow, U.K.). The oxygen electrode was calibrated on oxygenated saline ($pO_2$ = 150 mmHg; 1 mmHg = 133 Pa) and anoxic saline ($pO_2$ = 0 mmHg). Anoxic solutions contained 10 mM sodium sulfite and had been displaced with nitrogen gas for 20 min.

**Statistical Analysis.** The graphical package PRISM (GraphPad, San Diego) was used for statistical analysis, with ANOVA and $t$ test, and for plotting graphs.

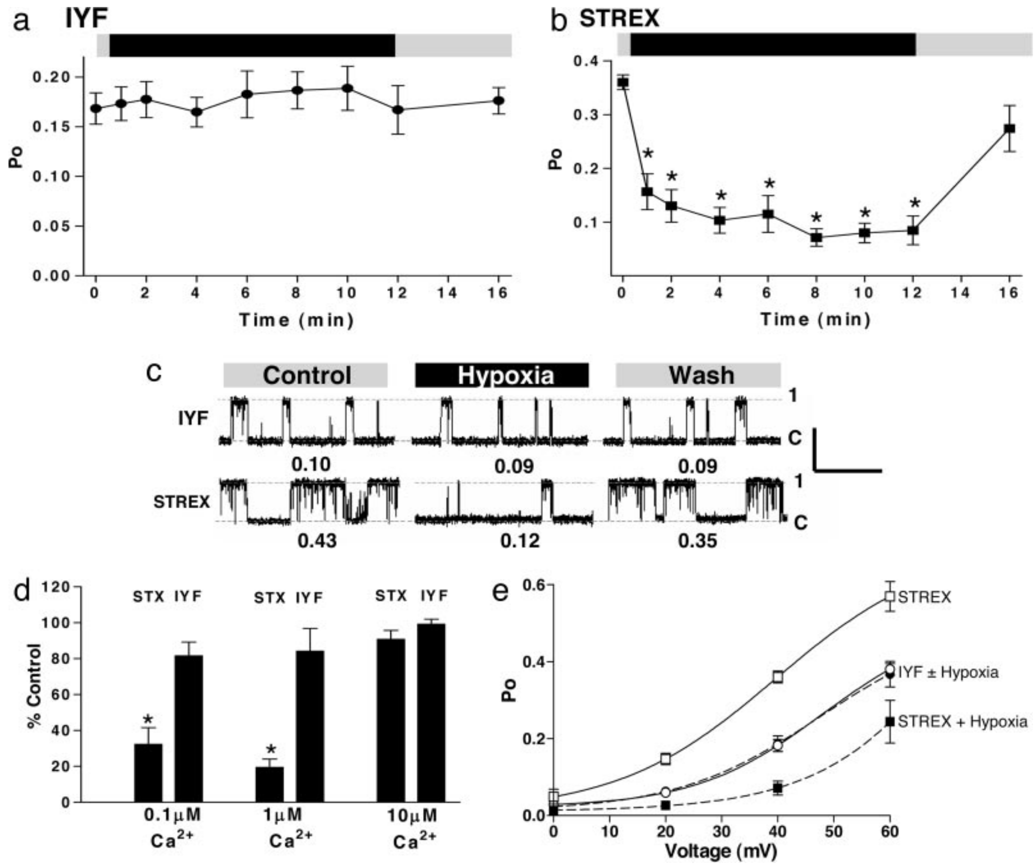


**Fig. 2.** Hypoxia inhibits the activity of the STREX channel variant expressed in HEK 293 cells. (*a*) Hypoxia (black bar) has no effect on IYF channel mean $P_o$ ($n$ = 12) in contrast to the potent inhibition of b STREX channels that was reversed upon wash out with normoxic saline (gray bar). (*a* and *b*) $P_o$ was determined at 40 mV in 1 $\mu$M $[Ca^{2+}]_i$. (*c*) Representative single-channel activity of IYF (upper trace) and STREX (lower trace) channels before, during, and after exposure to hypoxia. $P_o$ values are indicated. (Scale bar, 5 pA, 0.1 s.) (*d*) Hypoxia inhibits STREX channel $P_o$ (STX) at 0.1 and 1 $\mu$M $[Ca^{2+}]_i$ but not in 10 $\mu$M $[Ca^{2+}]_i$ ($n$ = 9–12 per group). (*e*) BK channel $P_o$ as a function of voltage in 1 $\mu$M $[Ca^{2+}]_i$. Hypoxia produced a significant right shift of the curve for the STREX variant (filled square and dotted line; $n$ = 12) when compared with values in normoxic saline (open square and continuous line; $n$ = 12). In the IYF variant, there was no significant shift when comparing activity in normoxic (open circle and solid line; $n$ = 12) or hypoxic solution (filled circle with dashed line; $n$ = 12). All data are given as mean ± SEM. *, $P < 0.01$, by ANOVA with post hoc test.

## Results

**Murine BK Channel $\alpha$-Subunit Splice Variants Are Differentially Sensitive to Hypoxia.** To investigate the importance of BK channel $\alpha$-subunit splice variants, we first assayed the hypoxia sensitivity of native BK channels in mouse anterior pituitary AtT20 corticotropes. These cells express three distinct $\alpha$-subunits that are alternatively spliced at the C-terminal C2 site of splicing: the insert-less ZERO variant; a variant with a 3-aa (IYF) motif; and the 58-aa cysteine-rich STREX variant; but lack $\beta_1$-subunit expression (27). Hypoxia ($pO_2$ = 18 ± 2 mmHg; $n$ = 9) potently and reversibly inhibited BK channels in isolated inside-out patches from AtT20 cells with a reduction of 65–80% in channel mean $P_o$ compared with pretreatment normoxic ($pO_2$ = 156 ± 4 mmHg; $n$ = 9) controls (Fig. 1 *a* and *b*). Hypoxia induced significant rightward shifts in the $V_{0.5max}$ in both 0.1 $\mu$M and 1 $\mu$M $[Ca^{2+}]_i$ from 52 ± 3 mV and 31 ± 3 mV in normoxia to 93 ± 3 and 76 ± 4 mV in hypoxia respectively ($P < 0.01$, by ANOVA with post hoc test; $n$ = 12–16). The voltage sensitivity of the channels, calculated as the potential required to produce an *e*-fold change in $P_o$, was not altered by the addition of hypoxic solution ($n$ = 12–16). BK channel activity was largely unaffected by hypoxia in the presence of 10 $\mu$M $[Ca^{2+}]_i$ (Fig. 1 *b* and *c*), suggesting that hypoxia sensitivity is Ca-dependent in agreement with several studies in other systems (1, 28).

To address whether the $\alpha$-subunit splice variants expressed in AtT20 cells are differentially sensitive to hypoxia, we expressed the ZERO, IYF, and STREX variants in HEK 293 cells. Hypoxia had no significant effect on single-channel conductance or activity of ZERO ($n$ = 4; data not shown) or IYF ($n$ = 16; see Fig. 2 *a* and *c*–*e*) variants at all investigated potentials. In contrast, under identical recording conditions, hypoxia significantly inhibited STREX variant mean channel $P_o$ upon exposure to hypoxia (Fig. 2 *a*–*d*). The magnitude of hypoxic inhibition at 40 mV in 0.1 $\mu$M $[Ca^{2+}]_i$ (68 ± 10%; $n$ = 14) was not significantly different from that observed in 1 $\mu$M $[Ca^{2+}]_i$ (78 ± 5% reduction in $P_o$; $n$ = 14) and was similar to that observed in AtT20 cells. Hypoxia caused a significant rightward shift in the STREX channel-activation curve in 0.1 and 1 $\mu$M $[Ca^{2+}]_i$ with $V_{0.5max}$ rising from 40 ± 3 mV to 92 ± 6 mV in 1 $\mu$M $[Ca^{2+}]_i$ ($P < 0.01$, by ANOVA with post hoc test; $n$ = 12) but did not alter the activation of the IYF channel (Fig. 2*e*). Hypoxia had no significant effect on STREX channel activity at 10 $\mu$M $[Ca^{2+}]_i$ in accordance with the Ca dependence that was observed in AtT20 cells ($n$ = 8; Fig. 2*d*). The lack of hypoxic response of STREX in 10 $\mu$M $[Ca^{2+}]_i$ and the lack of response of IYF channels at all Ca concentrations studied did not depend on the absolute value of channel $P_o$ (as shown in Fig. 2*e* for IYF and STREX). No inhibition of STREX channels was observed at 0 mV in 10 $\mu$M $[Ca^{2+}]_i$,

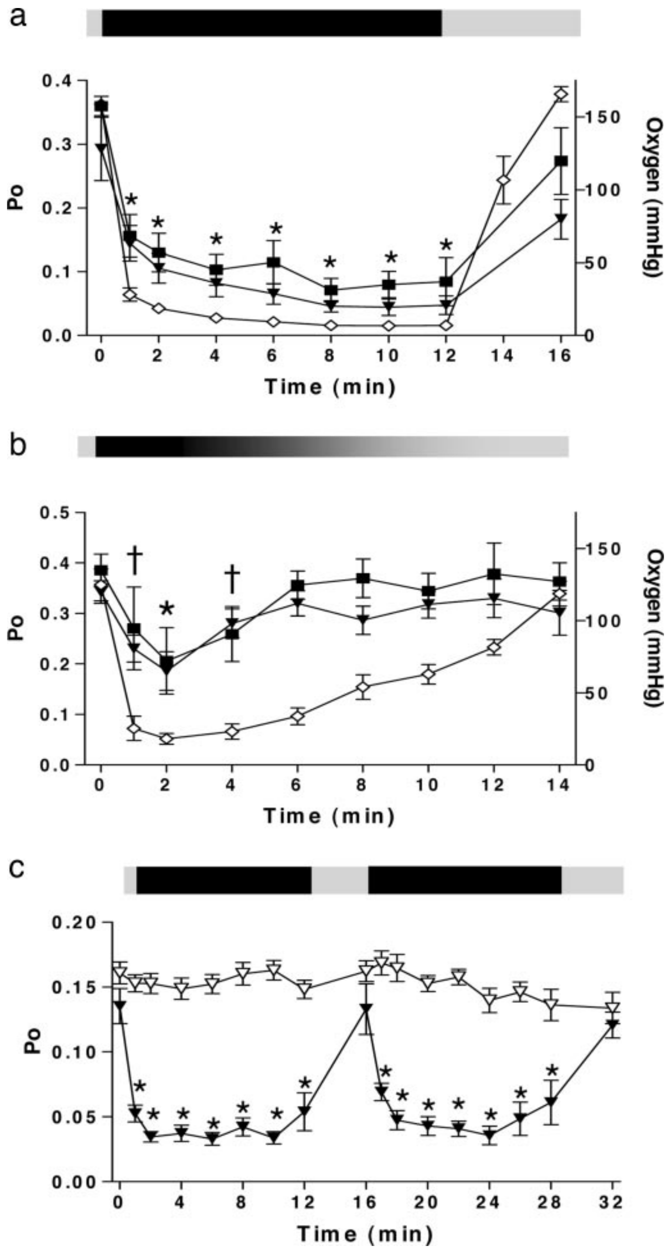


**Fig. 3.** Time course of hypoxic inhibition. The change in $P_o$ of BK channels in AtT20 cells (filled squares; $n = 12$) or STREX channels expressed in HEK cells (filled triangles; $n = 12$) plotted with the oxygen tension of the bath solution (open diamonds; $n = 9$) for a 12-min exposure to hypoxic saline (dark bar) followed by washout with normoxic saline (gray bar) determined at 40 mV and 1 $\mu$M $[Ca^{2+}]_i$ (*a*) and a brief 4-min exposure to hypoxic saline followed by slow requilibration to normoxia (graduated bar) (*b*). (*c*) Repeated exposure to hypoxic saline (black bars) inhibits STREX channels expressed in HEK cells (▼; $n = 12$). Normoxic saline failed to inhibit STREX channels (▽). Values were determined at 60 mV and 0.1 $\mu$M $[Ca^{2+}]_i$. Data are given as mean ± SEM. *, $P < 0.01$; and †, $P < 0.05$ (ANOVA with post hoc test).

where channel $P_o$ was 0.31 ± 0.03 ($n = 8$) similar to the $P_o$ at 40 mV in 1 $\mu$M $[Ca^{2+}]_i$. Hypoxia had no effect on single-channel conductance of STREX channels (126 ± 3 pS; $n = 16$) nor on the channel voltage sensitivity ($n = 16$).

Inhibition of both native AtT20 BK channels and STREX channels expressed in HEK 293 cells closely followed the change in oxygen tension in the bath solution as measured with an oxygen electrode (Fig. 3*a*). Significant channel inhibition was observed only during exposure to reduced oxygen tensions in the hypoxic range (i.e., <25 mmHg; Fig. 3*b*). Also, membrane exposure to a second hypoxic episode resulted in inhibition of channel activity identical in time course and magnitude as the first exposure to hypoxia (Fig. 3*c*), demonstrating that there was neither sensitization nor significant loss of function of the hypoxia-signaling mechanism. Because no exogenous substrates were applied, these data strongly support the hypothesis that the mechanism of inhibition is membrane delimited. Overall, these data indicate that functional channel regulation by hypoxia would only be manifest during markedly hypoxic episodes ($pO_2$ <25 mmHg), rather than during exposure to low but physiologically normal oxygen tension as measured in the CNS (7, 8) ($pO_2$ ≈ 50 mmHg).

**Sensitivity to Hypoxia Is Independent of Redox Regulation.** BK channels are sensitive to redox reagents (29–31), and the hypoxia sensitivity of STREX might arise from the significantly higher sensitivity of this $\alpha$-subunit splice variant to redox (32). The reducing agent sodium sulfite (1 mM) reversibly inhibited IYF, STREX, and AtT20 BK channels by 41 ± 5%, 77 ± 6%, and 57 ± 7%, respectively (at 40 mV in 1 $\mu$M $[Ca^{2+}]_i$; $n = 9$–14). Similarly, the cysteine-modifying agent *N*-ethylmaleimide (1 mM) inhibited IYF, STREX, and AtT20 BK channel activity by 53 ± 8%, 78 ± 6%, and 61 ± 8%, respectively (at 40 mV in 1 $\mu$M $[Ca^{2+}]_i$; $n = 9$–12). Although there was a difference in the size of the IYF and STREX channel responses to these agents, it was not possible to mimic the all-or-nothing response seen with hypoxia where STREX is largely (65–80%) inhibited, whereas there is no observable change in ZERO or IYF activity. Therefore, it is unlikely that redox sensitivity alone can explain the dramatic differences in responses to hypoxia between the BK channel splice variants.

**Sensitivity to Hypoxia Is Conferred by a Conserved CSC Motif in the STREX Insert.** The STREX, IYF, and ZERO variants used in these studies are identical apart from the distinct inserts at the C-terminal C2 site of splicing. Each variant contains the conserved heme binding motif (33) as well as the conserved cysteine residue close to the Ca bowl implicated in redox regulation (31), suggesting that these sites are not required for sensitivity to hypoxia. To investigate the differential sensitivity of the STREX and IYF (or ZERO) variants to hypoxia, we explored whether conserved cysteine residues (Fig. 4*a*) within the STREX insert are required for hypoxic inhibition. Cysteine-rich domains are important targets for oxidative regulation in many proteins, and recent structural analysis of protein tyrosine phosphatases revealed the important role of adjacent serine and cysteine residues (34, 35). Interestingly, a CSC motif is highly conserved from fish to man in the STREX insert (Fig. 4*b*) and does not occur in either ZERO or IYF. Although redox regulation *per se* does not explain the hypoxia sensitivity of STREX, we hypothesized that the CSC motif might confer intrinsic hypoxia sensitivity to BK channels. To test this hypothesis, we generated the following two mutant channels: STREX-C23A:C25A, in which both cysteines were mutated to alanine, and STREX-S24A, in which the serine residue was mutated to alanine.

The STREX-C23A:C25A and STREX-S24A mutants were efficiently expressed in HEK 293 cells. Hypoxia had no significant effect on these channels (Fig. 4 *c–e*) under identical conditions in which a robust inhibition of wild-type STREX and AtT20, BK channels was observed (Fig. 5). Even repeated exposure of STREX-C23A:C25A to hypoxic solution failed to induce a significant fall in activity; an 11 ± 10% inhibition ($n = 9$) was observed in 1 $\mu$M $[Ca^{2+}]_i$. This mutant retained redox sensitivity; channel inhibition to 1 mM sodium sulfite was 65 ± 9% ($n = 9$), which is not significantly different from STREX, suggesting that channel reduction does not underlie the hypoxic

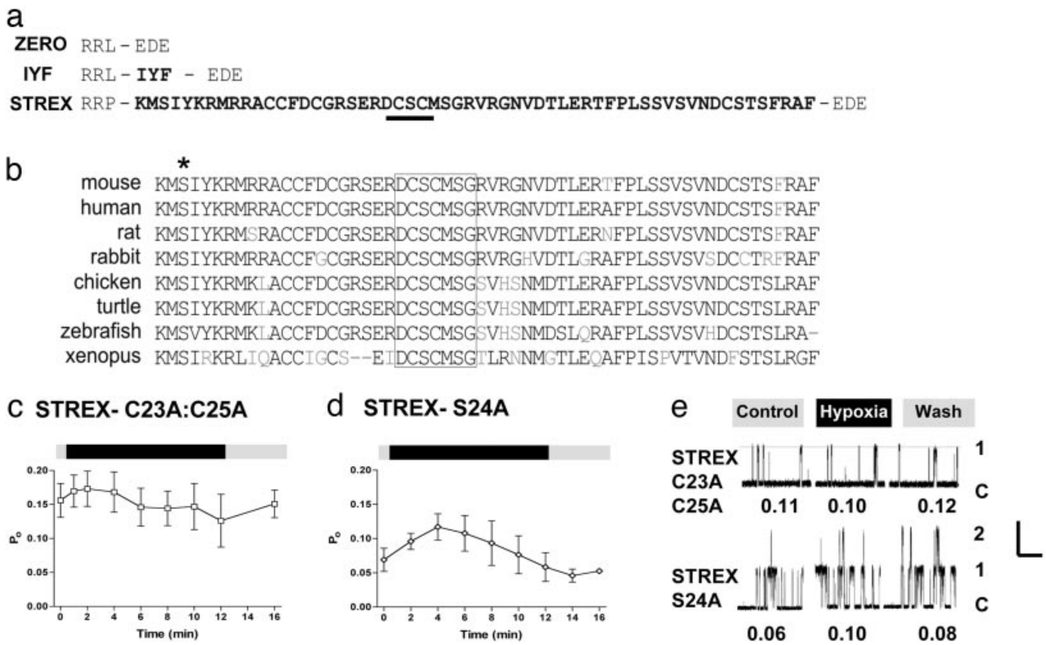


**Fig. 4.** Hypoxia sensitivity conferred by an evolutionary conserved CSC motif in the STREX insert. (*a*) Amino acid alignment of ZERO, IYF, and STREX at site C2, with the CSC motif underlined. (*b*) Alignment of STREX inserts across species. *, Serine available for PKA-dependent phosphorylation (21). Hypoxia had no effect on STREX-C23A:C25A (*c*; $n = 12$) or STREX-S24A (*d*; $n = 5$) channel activity. (*e*) Representative single-channel traces of STREX-C23A:C25A (upper trace) and STREX-S24A (lower trace) recorded at 40 mV in 1 and 0.1 $\mu$M $[Ca^{2+}]_i$, respectively, as in *b* and *c*. $P_o$ values are indicated. (Bar, 5 pA, 0.1 s.)

response. The difference in the response between STREX and the STREX mutants also did not depend on absolute channel $P_o$ (data not shown).

Because a CO-dependent mechanism has been proposed to underlie inhibition of human $\alpha + \beta_1$-subunit channel complexes by hypoxia (2), the absence of a response to hypoxia in the STREX mutant might reflect a loss of this CO sensitivity. The application of the CO donor (2), tricarbonyldichlororuthenium(II) dimer (50 $\mu$M), to STREX, STREX-C23A:C25A or STREX-S24A channels produced similar fold activation of 6.1 ± 1.2 ($n = 5$), 5.1 ± 1.4 ($n = 4$), and 4.8 ± 1.0 ($n = 4$), respectively. Together, these data suggest that the CSC motif in STREX is important for hypoxia regulation by means of a mechanism that is independent of redox or CO (2, 31, 33).

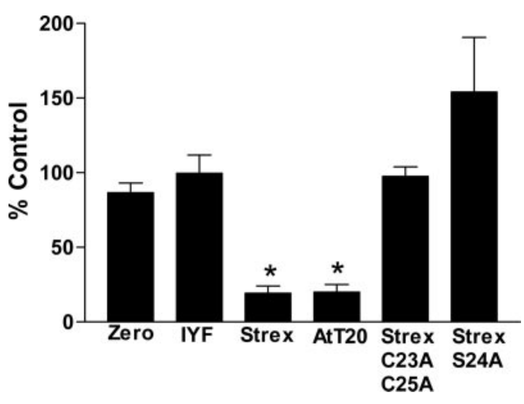


**Fig. 5.** Summary of the effect of hypoxia on the activity of BK channel splice variants and channel mutants. Summary of the effect of acute hypoxia on single-channel mean $P_o$ expressed as a percentage of the respective normoxic control for native (AtT20) and various recombinant BK channel $\alpha$-subunit splice variants and site-directed mutants expressed in HEK 293 cells. Data are given as means ± SEM. *, $P < 0.01$, by ANOVA with post hoc test.

## Discussion

We examined the response of BK channel $\alpha$-subunits to hypoxia to determine whether (*i*) BK channel pore-forming $\alpha$-subunits are sensitive to hypoxia in the absence of $\beta$-subunits, and (*ii*) alternative pre-mRNA splicing of $\alpha$-subunits provides a mechanism to generate functional diversity in BK channel responsiveness to changes in oxygen tension.

Our data demonstrate that distinct variants of murine BK channel $\alpha$-subunits display different sensitivity to exposure to hypoxia. We reveal a role for the alternatively spliced cysteine-rich STREX insert in conferring hypoxic inhibition to BK channel $\alpha$-subunits heterologously expressed in HEK 293 cells and to BK channels in AtT20 cells, which also express the STREX variant. Alternative pre-mRNA splicing provides a molecular on-off switch for BK channel sensitivity to hypoxia. The $\alpha$-subunits that lack an insert at the same site of splicing (ZERO), as well as those with a 3-aa insert (IYF), form channels that are completely insensitive to hypoxia, whereas STREX variants are potently inhibited by hypoxia. The inhibition of the STREX variant is Ca-dependent and reversible, and it rapidly follows the change in oxygen tension by means of a mechanism that is independent of redox (31) or CO (2) regulation. The Ca dependence of hypoxic inhibition has been observed (1, 28). Whether hypoxia modifies the Ca sensitivity of the channels or exerts its effect by means of other interactions remains to be determined. Hypoxic inhibition is abolished when a single, evolutionary conserved serine residue (S24) in the STREX insert, or the cysteine residues flanking S24 (C23 and C25 respectively), are mutated to alanine. Thus, a structural motif incorporating a serine residue flanked by a cysteine residue is important for sensitivity of BK channels to hypoxia. This effect may be analogous to the oxidative regulation of tyrosine protein phosphatases (34, 35). The mechanism by which the CSC motif transduces changes in oxygen tension to channel activity is unknown and requires further investigation, but is unlikely to result from a simple redox modification. The robust inhibition of the activity of the murine STREX $\alpha$-subunit splice variant by hypoxia (70–80%) in inside-out patches was similar to data described (2) using human BK $\alpha$- plus $\beta_1$-subunit coexpression in the presence of HO2 substrates. However, we observed this robust Ca-dependent, reversible, and repeatable hypoxic inhibition of the STREX variant in excised inside-out patches in the absence of exogenous HO2 substrates. Also, CO activated all tested $\alpha$-subunit splice variants, including the site-directed mutants in which hypoxic inhibition was abolished. Thus, our data reveal a HO2/CO-independent mechanism that is most likely to be intrinsic to the STREX $\alpha$-subunit splice variant rather than to depend on secondary signaling systems. These data support the hypothesis that several distinct mechanisms of hypoxic regulation of BK channels exist (i.e., both HO2/CO-independent and -dependent mechanisms; ref. 2).

It is not known whether the coexpression of the $\beta_1$-subunit is obligatory for the HO2/CO-dependent mechanism (2) or may modify the sensitivity by means of the HO2/CO-independent pathway. Previous studies have demonstrated, by using a human $\alpha$-subunit variant that lacks the STREX insert coexpressed with the $\beta_1$-subunit, a modest (≈30%) inhibition via an HO2/CO-independent pathway (28). We detected no effect of hypoxia on ZERO or IYF $\alpha$-subunits expressed in the absence of $\beta_1$-subunits. Although $\beta_1$-subunits are widely expressed in cells of the vasculature, many tissues that display hypoxia sensitive BK channels, such as neurones and many endocrine cells, do not express $\beta_1$-subunits (4, 27). Thus, together with our data on robust inhibition of STREX variant $\alpha$-subunits, $\beta_1$-subunits are not required for hypoxic regulation of BK channels *per se.* The existence of several independent mechanisms for hypoxic regulation of BK channels (including modulation by redox agents, the heme-dependent pathways, and the splice-variant-specific pathway described here) provides cells with different pathways to monitor changes in oxygen tension. Thus, the relative contribution of these mechanisms for hypoxic regulation of BK channels is likely to be different between cell types, according to the make-up of endogenous channels and the physiological demands of the cell.

Alternative splicing of the BK channel pore-forming $\alpha$-subunit provides cells and tissues with a mechanism to specify sensitivity to hypoxia. Because STREX variant expression is tissue-specific and can be dynamically regulated in adults (18, 19, 36, 37), splicing may provide an adaptive mechanism to match cellular excitability and Ca entry, which BK channels modulate, to physiological requirements. Thus, dynamic control of STREX variant alternative splicing (18, 19, 36) would provide a mechanism to generate tissue-specific sensitivity and plasticity of cellular responses to hypoxia.

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