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Hypoxia Regulates Expression and Activity of Kv1.3 Channels in T Lymphocytes: A Possible Role in T Cell Proliferation

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T lymphocytes are exposed to hypoxia during their development and also when they migrate to hypoxic pathological sites such as tumors and wounds. Although hypoxia can affect T cell development and function, the mechanisms by which immune cells sense and respond to changes in O2-availability are poorly understood. K+ channels encoded by the Kv1.3 subtype of the voltage-dependent Kv1 gene family are highly expressed in lymphocytes and are involved in the control of membrane potential and cell function. In this study, we investigate the sensitivity of Kv1.3 channels to hypoxia in freshly isolated human T lymphocytes and leukemic Jurkat T cells. Acute exposure to hypoxia (20 mmHg, 2 min) inhibits Kv1.3 currents in both cell types by 20%. Prolonged exposure to hypoxia (1% O2 for 24 h) selectively decreases Kv1.3 protein levels in Jurkat T cells by 47%, but not in Kvβ2 and SK2 Ca-activated K+ channel subunit levels. The decrease in Kv1.3 protein levels occurs with no change in Kv1.3 mRNA expression and is associated with a significant decrease in K+ current density. A decrease in Kv1.3 polypeptide levels similar to that obtained during hypoxia is produced by Kv1.3 channel blockage. Our results indicate that hypoxia produces acute and long-term inhibition of Kv1.3 channels in T lymphocytes. This effect could account for the inhibition of lymphocyte proliferation during hypoxia.

Indeed, we herein present evidence showing that hypoxia selectively inhibits TCR-mediated proliferation and that this inhibition is associated with a decrease in Kv1.3 proteins. The Journal of Immunology, 2003, 170: 695–702.

Low oxygen availability (hypoxia) can be encountered by immune cells both in physiological and pathological conditions. Immune cells are exposed to various oxygen tensions (PO2) as they mature and migrate in different tissues (1). Whereas arterial blood has a PO2 of ~90–100 mmHg and normal tissues typically have a PO2 in the range of 40–60 mmHg, oxygen tensions as low as 4 mmHg have been measured in lymphoid organs (1). Moreover, immune cells reach pathological sites such as tumors, wounds, and inflamed areas where severe hypoxia (PO2 < 10 mmHg) has been reported (2–4). Thus, hypoxia might affect normal immune cell development as well as the function of immune cell activity at hypoxic pathological sites. Indeed, various in vitro studies have indicated that hypoxia can affect the function of the host immune cells (5–9). Hypoxia has been shown to markedly diminish lymphocyte expression of IL-2, proliferation, and to impair the activity of NK cells (7, 8, 10). Recently, Caldwell et al. (1) has shown that hypoxia can alter lymphphokine secretion pattern and CTL development. Still, although hypoxia can have such profound effects on immune cell function, the mechanisms by which immune cells respond and adapt to hypoxia are poorly understood (11). However, the downstream effects of hypoxia on nonimmune cells, in particular chemosensitive cells, have been extensively studied (12). It is now well established that one of the immediate cellular responses to hypoxia is inhibition of O2-sensitive K+ (Ko2) currents. This inhibition leads to membrane depolarization, changes in intracellular Ca2+ concentration ([Ca2+]i), and ultimately, it affects cell function (12). In many O2-sensitive cells such as carotid body type I cells, pulmonary artery smooth muscle cells, and pulmonary neuroepithelial body cells, the Ko2 channel appears to belong to the family of voltage-dependent K+ (Kv) channels (13). Kv channels are tetrameric arrangements of four separate pore-forming α proteins and auxiliary Kvβ subunits (14). The genes that encode the Kvα subunits have been classified in four major subfamilies: Kv1–Kv4. New subfamilies (Kv5–Kv10) have been recently added. The Kvα subunits implicated in forming Ko2 channels are: Kv2.1 (homomultimer or heteromultimer in combination with the silent Kv9.3α subunit), Kv1.5, Kv1.2, Kv3.1b, and Kv4.2 (12). Hypoxia exerts two effects on Ko2 channels: an acute effect occurs within minutes, consisting of inhibition of the channel activity; a chronic effect occurs after prolonged exposure to hypoxia, which is associated with down-regulation of the channel itself (12). Although much progress has been made in identifying the different Ko2 channels in different chemosensitive cells, the mechanisms mediating their oxygen sensitivity are not fully understood. Moreover, the expression of these channels in other cell types is still to be determined.

Lymphocytes express voltage-dependent (Kv) and Ca-activated K (K(Ca)) channels and their activity is essential in T cell activation. In fact, K+ channels indirectly regulate the Ca2+ signal necessary for cell proliferation and cytokine production by modulating the resting potential of T cell membrane (15). The Kv channel expressed in lymphocytes is a member of the Kv1 family of Kv channels, specifically Kv1.3. These channels are highly expressed in resting human T lymphocytes and blockade of Kv1.3 channels suppresses cytokine production and cell proliferation (15, 16).

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3 Abbreviations used in this paper: Ko2, O2-sensitive K+; [Ca2+]i, Ca2+ concentration; Ionom, ionomycin; 4-AP, 4-amino pyridine; MgTX, margatoxin; CRAC, calcium release-activated channel; OKT3, immobilized anti-CD3 Ab; Kv, voltage-dependent K+; K(Ca), Ca-activated K+; Ile, K+ current; Ile, current density.
Kv1.3 channels are also important in the regulation of cell volume and lymphocyte adhesion and migration (17, 18).

Although various studies have been performed to study the mechanisms of lymphocyte adaptation to hypoxia (11, 19), no information is available as to whether other elements important in sensing changes in O2 availability, such as the O2-sensitive K (Ko3) channels, are expressed in immune cells. The present study was undertaken to investigate the sensitivity to hypoxia of Kv1.3 channels in human T lymphocytes.

Materials and Methods

Cells

Jurkat T cells (clone E6-1) were obtained from American Tissue Culture Collection (Rockville, MD). PBMC were obtained from consenting healthy adult donors. Venous blood was collected in heparinized tubes and diluted 50% with balanced salt solution (in millimolars: Tris 145, CaCl2 0.05, MgCl2 1 mM, KCl 5.4, NaCl 140, glucose 0.1% (pH 7.6). The suspension was centrifuged at 400 g for 4 min through a Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density gradient for 30 min at room temperature. The interface was removed, washed with balanced salt solution, and resuspended in RPMI medium (10% FBS and 100 U/ml penicillin, and 100 µg/ml streptomycin). Adherent cells were removed by incubation in petri dishes for 2 h at 37°C. PBMC were cultured at an initial density of 1 × 106 cells/ml. FACs analysis of PBMC showed that CD3+ lymphocytes constitute ~80% of the peripheral blood lymphocytes in the PBMC population (data not shown). Resting and activated freshly isolated lymphocytes were used for our electrophysiological experiments. Activation was accomplished by incubating the lymphocytes with PHA (4 µg/ml) for at least 24 h. In some experiments, T cells were separated from PBMC using the E-rosette technique (Stem Cell Technologies, Vancouver, Canada). FACs analysis showed that 98% of the cells isolated by E-rosetting were CD3+ (data not shown). Jurkat and freshly isolated lymphocytes were grown in RPMI (supplemented as above) and maintained in a humidified incubator at 37°C.

Electrophysiology

K+ currents were recorded in either whole-cell or perforated patch configurations. In these experiments, the external solution had the following composition (in millimolars): 150 NaCl, 5 KCl, 2.5 CaCl2, 1.0 MgCl2, 10 glucose, and 10 HEPES, pH 7.4. The pipette solution was composed of (in millimolars): 134 KCl, 1 CaCl2, 10 EGTA, 2 MgCl2, 5 ATP-sodium, and 10 HEPES, pH 7.4, with an estimated free [Ca2+] of 10 nM (20). Recordings were performed on resting and activated freshly isolated lymphocytes with a membrane capacitance of 2.0 ± 0.6 pF (n = 14) and 3.4 ± 0.3 pF (n = 15; p < 0.05), respectively. Similar capacitance has been reported for resting and activated human T cells (21). During our electrophysiological experiments, we visually excluded monocytes and phagocytic cells and selectively chose lymphocytes by their appearance and small diameter (6–8 µm, ~2 pF) (22). Resting CD3+ cells isolated by E-rosetting have similar capacitance (1.9 ± 0.4 pF n = 7; p = 0.8) and current density (I0; 300 ± 99 pApF for CD3+ obtained by E-rosetting, n = 7, and 208 ± 74 pApF, n = 14, in resting lymphocytes visually selected from the PBMC population, p = 0.5). Jurkat cells had a membrane capacitance of 11.7 ± 1.0 pF (n = 31). K+ currents were recorded in voltage-clamp mode by depolarizing voltage steps to +50 mV (800 ms duration, every 30 s) from a holding potential of ~80 mV. The temperature in the patch-clamp chamber was stable at 23 ± 0.1°C (n = 6) both in normoxic and hypoxic solutions, as it is known that Kv1.3 channels are highly sensitive to changes in temperature (23). To obtain the perforated patch configuration, nystatin (Molecular Probes, St. Louis, MO) was dissolved before use in DMSO (Fisher Scientific, Fair Lawn, NJ) at a concentration of 50 mg/ml and added to the pipette solution to a final concentration of 150–200 µg/ml. Nystatin solutions were used within 2 h after preparation. The pipette tip was dipped in the intracellular solution, then backfilled with nystatin solution. Experiments were performed using Axopatch 200A amplifier (Axon Instruments, Foster City, CA). The digitized signals were stored and analyzed using pClamp 8 software (Axon Instruments).

Exposure of cells to hypoxia

During electrophysiological experiments, the effect of acute hypoxia was studied by switching from a perfusion medium bubbled with air (21% O2) to a medium equilibrated with 100% N2. A polarographic oxygen electrode (World Precision Instruments, Sarasota, FL) placed in the perfusion chamber was used to monitor the PO2 in the bath solutions. A complete exchange of PO2 in the perfusion chamber was reached within 60 s. To study the effect of prolonged hypoxia on protein and gene expression, cells were maintained in 1–5% O2 (~8–40 mmHg) for the entire course of an experiment (24 h) in an incubator with controlled PO2 (Thermo Forma, Marietta, OH). To study the effect of hypoxia on T cell proliferation, cells were maintained in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) at 37°C, in an atmosphere saturated with 1% O2.

Cell viability assay

At the end of chronic hypoxia experiments, cell viability was determined by trypan blue exclusion. Cells were then pelleted by centrifugation (1,400 rpm for 2 min) and resuspended in normal Tyrode solution (in millimolars: NaCl 143, KCl 5.4, MgCl2 0.5, CaCl2 1.8, HEPES 5, glucose 5.5, pH 7.4). Equal volumes of cell suspension and 0.4% trypan blue (Sigma-Aldrich) were mixed and incubated at room temperature for 5 min. Cells were then counted using a hemocytometer. Cell viability (percentage) was determined as the ratio of total viable cells (unstained)/total cells (stained and unstained). The data reported are the average of separate experiments, each in quadruplicate.

Western blotting

Jurkat T cell total lysate was prepared according to standard procedures. Human fresh lymphocytes total lysate was prepared from Buffy Coat. Cells were harvested by centrifugation (1,400 rpm for 2 min) and resuspended in lysis buffer consisting (in millimolars): 10 Tris, 1 EDTA, 1 PMSF, and 1% Triton X-100. Cells were then washed twice in 40 mM sodium phosphate buffer, pH 7.2, and 208 g/ml leupeptin, and 2 µg/ml aprotinin. After sonication and centrifugation, the protein content was measured using the BCA kit (Pierce, Rockford, IL). Aliquots of cell proteins were fractionated on 6% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific protein-binding sites were blocked by incubation in TBST with 5% nonfat dry milk for 1 h at room temperature. The blots were incubated for another 1 h with the primary antibody (1/400 for anti-SK2, and 1/1600 for anti-B2 Abs) overnight at 4°C. After washing three to four times, the membranes were incubated for 1 h at room temperature with affinity-purified HRP-conjugated secondary Abs (Pierce and Alphadiagnostic International, San Antonio, TX). Bands were visualized using SuperSignal Chemiluminescence (Pierce) and quantitated by densitometric analysis using UNSCAN-IT gel software (SilkRoad Technologies, CA). Prestained m.w. standards were used to assess the apparent m.w. Anti-Kv1.3 and anti-SK-2 Abs were obtained from Alomone Labs (Jerusalem, Israel). Anti-Kv shaker β-2 (anti-Kvβ2) and anti-β-actin Abs were obtained from BioSource International (Camarillo, CA) and Alphadiagnostic International, respectively.

Northern blot hybridization

Total RNA was isolated from Jurkat cells with TRI REAGENT (Molecular Research Center, Cincinnati, OH), according to the manufacturer’s protocol (24). Total RNA samples (30 µg/lane) were fractionated on a 1.2% agarose-formaldehyde gel and transferred to nylon membranes using 10X sodium chloride–sodium phosphate–EDTA as transfer buffer. Membranes were cross-linked by UV light and baked for 1 h. Hybridization was performed according to standard procedures. Briefly, membranes were placed for 1 h in 0.1X sodium chloride–sodium phosphate–EDTA/1% SDS solution at 65°C. The membranes were then prehybridized for 10 min at 65°C with 0.5 M sodium phosphate buffer, pH 7.2, 7% SDS, 1% BSA, and 1 mM EDTA. Thereafter, the membranes were hybridized overnight in the above solution with 30–50 × 106 cpm of [32P] labeled Kv1.3 cDNA probe obtained by PCR of the full-length cDNA clone. The membranes were washed twice in 40 mM sodium phosphate buffer, pH 7.2, 5% SDS, 0.5% BSA, and 1 mM EDTA for 10 min at 65°C, washed four times in 40 mM sodium phosphate buffer, pH 7.2, 1% SDS, and 1 mM EDTA for 10 min at 65°C, exposed to PhosphorImager cassettes at room temperature for 24–72 h, and read by a PhosphorImage (Molecular Dynamics, Sunnyvale, CA). Bands were quantitated by densitometric analysis (Image-Quant 5.0; Molecular Dynamics).

Proliferation assay

Resting PBMC were seeded at 2 × 106 cells/well in culture medium in flat-bottom 96-well plates (final volume 200 µl). Cells were cultured for 2 h in normoxia or hypoxia (1% O2), after which the following proliferating agents were added: 25 µg/ml PHA, 167 µg/ml Con A, 25 µg/ml OKT3 (immobilized anti-CD3 Ab), 0.5–2 µg/ml ionomycin (Ionom) (with or without PMA (PMA/Ionom = 10 ng/ml/0.5 µg), 20 µg/ml candida (Greer Labs, Lenoir, NC), and 0.025–0.1 U/ml tetanus (Aventis Pasteur, Lyon, France). The cells were then returned to either the normoxic or hypoxic atmosphere for the remaining of the experiment. Fourteen hours before
hunting. [1H]thymidine (2 μCi/well) was added. The cells were harvested onto glass fiber filters, [1H]thymidine incorporation was measured in a scintillation counter.

Data analysis
All data are presented as means ± SEM. Statistical analyses were performed using Student’s t test (paired or unpaired); p ≤ 0.05 was defined as significant.

Chemicals
4-Amino pyridine (4-AP), PHA, Con A, Ionom, and PMA were obtained from Sigma-Aldrich. OKT3 was obtained from Ortho Biotech (Toronto, Ontario, Canada). Margatoxin (MgTX) was purchased from Alomone Labs.

Results
Hypoxic inhibition of Kv1.3 channel activity in human lymphocytes
Electrophysiological experiments were performed to study the effect of acute hypoxia on Kv1.3 channel activity in lymphocytes. Acute hypoxia was produced by switching from a perfusion medium bubbled with air (21% O2) to a medium equilibrated with 100% N2. The mean PO2 values measured in the recording chamber were 133 ± 1.5 mmHg (air, n = 8) and 20 ± 1 mmHg (100% N2, n = 8; p < 0.0001). A representative actual PO2 measurement in the recording chamber is Fig. 1A, inset. Outward K+ currents (IK) were recorded in lymphocytes in whole-cell voltage-clamp experiments. Kv1.3 currents were isolated by dialyzing the cells with a nominally Ca2+-free pipette solution which allows minimizing of the contribution of Ca2+-activated K+ (K(Ca)) channels to the total current (25). The pipette solution also contained ATP to compensate for reduced cellular energy metabolism during hypoxia. Acute hypoxia (~20 mmHg) inhibits Kv1.3 currents in fresh human lymphocytes (Fig. 1A). Introduction of hypoxia rapidly inhibited IK peak amplitude by 20.1 ± 3.0% from 690.8 ± 145.6 pA in normoxia to 554.2 ± 113.6 pA in hypoxia (n = 11, p < 0.005; Fig. 1A, B, and D). The inhibitory effect of hypoxia was displayed by 80% of the cells tested. A similar degree of inhibition was observed in resting and activated lymphocytes; 19.7 ± 6.5% (n = 4) in resting and 20.4 ± 3.3% (n = 7) in activated (p = 0.9). The inhibition of Kv1.3 peak current amplitude by hypoxia was immediate, reversible, and reproducible (Fig. 1B). Inhibition of similar amplitude was produced by a second exposure to hypoxia (21.7 ± 6.2%, n = 3; p = 0.8 vs first exposure). This response was confirmed in human CD3+ cells separated from PBMC by E-rosetting. In these cells, hypoxia inhibited IK peak amplitude by 19.3 ± 8.4% (n = 3). Furthermore, a similar response was produced in Jurkat T cells. Hypoxia inhibited peak IK of 19.7 ± 5.4% from 1027.4 ± 292.5 pA in normoxia to 864.9 ± 275.6 pA after 2 min in hypoxia (n = 7; p < 0.05). Hypoxia inhibition was observed in 64% of the cells tested and it was observed in both whole-cell and nystatin patch experiments: 19.0 ± 3.4% (n = 3) and 20.2 ± 9.8% (n = 4), respectively.

Down-regulation of Kv1.3 expression in T lymphocytes by chronic hypoxia
T cells respond immediately to acute hypoxia with inhibition of the Kv1.3 current. This response is characteristic of O2-sensitive K channels (12). There are many examples that the expression of these K02 channels in other cell types is regulated during prolonged exposure to hypoxia (26–28). Therefore, we evaluated the effect of chronic hypoxia on Kv1.3 expression in Jurkat cells maintained in 1% O2 (~8 mmHg) for 24 h. The Kv1.3 protein levels in hypoxic cells were compared with those measured in cells maintained in normoxia and cells exposed to 8.6 mM 4-AP (a K channel blocker) in normoxia for 24 h. This concentration of 4-AP has previously been shown to inhibit Kv current and mitogenesis in human T lymphocytes without affecting cell viability (29). The Kv1.3 polypeptide in total lysate of Jurkat cells was detected with the affinity-purified Ab anti-Kv1.3 (Fig. 2). Western blot analysis revealed a single band of ~58 kDa. This band disappeared when the Ab was preabsorbed to the corresponding Ag (data not shown), thus indicating specificity of the anti-Kv1.3 Ab. A band of similar size has been identified as an α subunit for Kv1.3 (30). Kv1.3 protein expression significantly decreased after 24 h of exposure to 1% O2 (H; Fig. 2). No decrease in cell viability was observed in Jurkat cells maintained 24 h in hypoxia: the percentage viability, as determined by trypan blue exclusion, was 86 ± 1% in normoxia and 93 ± 1.4% in hypoxia (n = 2). The Kv channel inhibitor 4-AP under normoxic conditions also produced a decrease in Kv1.3 protein levels. The hypoxic down-regulation of Kv1.3 protein expression depended on the severity of hypoxia (Fig. 2C). Exposure to 5% O2 (~40 mmHg) produced a significant 10% decrease in protein expression from 100.0 ± 1.3% in normoxia (n = 4) to 89.6 ± 1.9% in hypoxia (n = 4; p = 0.004), while a 47% inhibition was obtained with more severe hypoxia (~8 mmHg, see above). Neither hypoxia nor MgTX (a specific blocker of Kv1.3) (31) regulated Kv1.3 gene expression (Fig. 3).

To determine the specificity of Kv1.3 hypoxic inhibition, we examined the effect of hypoxia on the expression of other K+ channels present in Jurkat cells. Lymphocytes are known to express both Kv and Ca2+-activated K (K(Ca)) channels. A small conductance K(Ca) channel encoded by hSK2 gene is abundant in the human leukemic Jurkat T cell line (25). Western blot experiments similar to those described above were performed to study the effect of chronic hypoxia (1% O2 for 24 h) on SK2 expression in Jurkat cells (Fig. 4). Anti-SK2 Ab recognized a band of 57 kDa. A band of similar size has been identified as SK2 (32). In contrast...
to Kv1.3, SK2 expression was not modified by either hypoxia or 4-AP. This indicates that hypoxia selectively down-regulates Kv1.3/H9251 subunit expression.

Decreased expression of functional Kv1.3 channels by chronic hypoxia

Whole-cell voltage-clamp experiments were performed to determine whether the decrease in Kv1.3 α subunit expression produced by chronic hypoxia translated into an actual decrease in functional Kv1.3 channels. Consistent with the Western blot experiments, chronic hypoxia significantly reduced Kv1.3 Iₚ as compared with that observed in Jurkat cells maintained in normoxia (Fig. 5). It is possible that this decrease in expression of functional Kv1.3 channels by chronic hypoxia may produce long-term effects on T cell activity.

Factors other than Kv1.3 α subunit expression can contribute to the decreased Kv1.3 Iₚ produced by chronic hypoxia. Functional Kv channels are heteromultimeric complexes of the pore-forming α subunits and auxiliary Kv β subunits (14). The latter subunits are important to promote Kv channel surface expression and to stabilize the channel complexes (33). Thus, to establish a possible contribution of Kvβ2 subunits in the decreased Kv1.3 Iₚ during hypoxia the expression of Kvβ2 subunits was determined in normoxic and hypoxic conditions. The results showed that chronic hypoxia significantly reduced the expression of Kvβ2 subunits, as determined by Western blot analysis.

![Figure 4](http://www.jimmunol.org/Downloadedfrom/698_DOWN-REGULATION_OF_Kv1.3_EXPRESSION_DURING_CHRONIC_HYPOXIA.png)

**FIGURE 4.** Effect of chronic hypoxia on SK2 protein expression. A, Western blot analysis of SK2 protein in Jurkat cells cultured in normoxia (N; lanes 1-3) and in hypoxia (H, 1% O₂, ~8 mmHg; lanes 4-6) for 24 h. B, Immunoblot analysis of SK2 protein in Jurkat cells cultured in normoxia (N) with (lanes 5-7) and without (lanes 1-4) the Kv blocker 4-AP (8.6 mM) for 24 h. A total of 40 μg total lysate was loaded in each lane. C, Averaged densitometric analysis of SK2 normalized to β-actin in Jurkat cells cultured in normoxia (N, n = 3) and in hypoxia (H, n = 3), and in normoxic cells in presence of 4-AP (n = 3). Data are expressed as percentage of control (normoxia).
subunits, the levels of Kvβ2 polypeptide do not change during hypoxia.

**Hypoxia selectively inhibits proliferation generated by TCR cross-linking**

It is well established that blockers of Kv channels are able to inhibit T cell proliferation (15). Hypoxia was also shown to inhibit lymphocyte proliferation (1, 10). We have studied the effect of hypoxia on T cell proliferation and we have compared its effect on proliferation resulting from different mitogenic stimuli. Proliferating agents used were: either 1) agents that induce cell activation by cross-linking the TCR, such as PHA, Con A, OKT3, and Ags such as candida and tetanus presented by APCs; or 2) agents that induce proliferation bypassing the membrane such as Ionom (a calcium ionophore), with and without PMA. The difference between these two groups is that the former relies on the influx of Ca\(^{2+}\) through the calcium release-activated channel (CRAC) channels (whose activity depends on the Kv1.3 channels) while the latter produces an increase in intracellular [Ca\(^{2+}\)] independently of membrane channels. Viability of the cells was estimated at the end of each experiment. No change in viability was observed between normoxic and hypoxic cells (95 ± 2 and 96 ± 1%, n = 14, after up to 7 days in normoxic and hypoxic conditions, respectively). Our data show that hypoxia selectively inhibits T cell proliferation induced by agents that cross-link the TCR (Fig. 7A). Proliferation induced by stimuli that bypassed the membrane was not affected by hypoxia. This is expected if the decrease in proliferation during hypoxia is mediated by the hypoxic inhibition of the Kv1.3 channels. Thus, we have measured the levels of Kv1.3α subunits in the

 aberrantly hypoxic T lymphocytes. Anti-Kvβ2 polyclonal Ab recognized a band of the predicted molecular size (39 kDa) in Jurkat cell total lysate (Fig. 6). This 39-kDa protein is identical in size to the Kvβ2 channel subunit purified from bovine brain (34) and the Kvβ2 subunit detected in human T lymphocytes (35). A faint band just below the 39-kDa band is likely to correspond to a previously reported splice variant of Kvβ2 (35). In contrast to the Kv1.3 α
protein lysates obtained from hypoxic and normoxic T lymphocytes after 48–72 h stimulation with PHA. We found that the levels of Kv1.3 α subunit proteins were significantly decreased during hypoxia by 46 ± 13% (n = 4, p = 0.01) (Fig. 7B).

**Discussion**

In the current manuscript, we have presented evidence that hypoxia regulates Kv1.3 channels in T lymphocytes. Hypoxia exhibits a dual effect on these channels depending on the duration of the hypoxic exposure: acute hypoxia inhibits Kv1.3 channel activity while chronic hypoxia down-regulates the expression of functional Kv1.3 channels. The latter effect is due to the selective decrease in Kv1.3 protein levels during hypoxia. Thus, overall our findings indicate that the main Kv channels expressed in T cells are sensitive to hypoxia. K⁺ channels sensitive to changes in oxygen availability (Kₒ₂ channels) are expressed in various chemosensitive cells (12). To our knowledge, this is the first time that a Kₒ₂ channel has been described in T lymphocytes.

**Inhibition of Kv1.3 channel activity by acute hypoxia**

The acute effect of hypoxia on Kv1.3 channels in T lymphocytes resembles the effect described for other Kₒ₂ channels in other cell types (24, 36–38). Kₒ₂ channels are rapidly inhibited by hypoxia and their hypoxic inhibition occurs without known modifications in cytosolic pH, [Ca²⁺], and ATP (12). Our experiments have shown that exposure to decreased PO₂ produces an immediate inhibition of Kv1.3 current in T cells, reaching steady state within 2 min from exposure. The inhibitory effect of hypoxia is observed under conditions of constant intracellular ATP levels, pH, and [Ca²⁺] as they are maintained by dialyzing the cell with a pipette solution of known composition during whole-cell voltage-clamp experiments.

The ability of Kₒ₂ channels to immediately respond to hypoxia had placed them in a key position in the process of O₂ sensing, and it was even speculated that the Kₒ₂ channel itself could function as an O₂ sensor (39). The mechanisms by which mammalian cells sense changes in O₂ availability and the nature of the O₂ sensor are still under debate (12, 39). Changes in intracellular redox state as well as membrane-associated events have been implicated in mediating the hypoxic response (12). It is possible that distinct molecular mechanisms may mediate the regulation of different Kv channels by hypoxia in different cells. Recently, Cakabayab et al. (30) have shown that anoxia together with glucose deprivation inhibits a Kv1.3 current in rat microglia (immune-competent cells present in the central nervous system). This inhibition was mediated by src tyrosine kinases. It has also been shown that recombinant and native Kv1.3 channels are inhibited by hydrogen peroxide (H₂O₂) and it was proposed that this effect is due to direct oxidation of these channels as it was independent of src-like tyrosine kinase phosphorylation and was mimicked by thiol group-oxidizing agents (20, 40). In contrast to the hypoxic inhibition of Kv1.3 channels, the effect of H₂O₂ occurs over a much longer time frame and is irreversible. Therefore, it appears that different mechanisms underlie hypoxia- and H₂O₂-mediated Kv1.3 inhibition. Additional experiments are underway to determine the mechanisms mediating Kv1.3 current inhibition by hypoxia in T cells.

**Selective down-regulation of functional Kv1.3 channels during chronic hypoxia**

Chronic hypoxia reduces the expression of functional Kv1.3 channels in T cells by down-regulating the protein expression of the pore-forming Kv1.3 α subunit. The inhibitory effect of hypoxia is specific to this channel since the expression of SK2 channel subunit or the Kv channel regulatory β subunit Kvβ2 is not affected. The expression of functional Kₒ₂ channels in other cell types is regulated by chronic hypoxia (24, 28). In particular, chronic exposure to hypoxia reduces Kₒ₂ current in pulmonary artery smooth muscle cells by down-regulating the mRNA and protein expression of Kv1.5 and Kv2.1 α subunits (28). Interestingly, we have observed that hypoxia does not induce a decrease in steady-state levels of Kv1.3 mRNA in Jurkat T cells, suggesting that different mechanisms are likely to mediate the down-regulation of Kₒ₂ channels in different cell types. There is evidence that regulation of Kv1.3 protein occurs posttranscriptionally, independent of changes in RNA expression (41). In fact, the increase in Kv1.3 channels that occurs in activated T cells is not accompanied by an increase in its mRNA; on the contrary, recent gene array data have shown that activation produces a decrease in Kv1.3 mRNA (42). Our data suggest that down-regulation of Kv1.3 channel expression by hypoxia also occurs posttranscriptionally. There are other examples of Kv channel regulation occurring at the protein level: in PC12 cells, Kv2.1 protein levels are increased by exposure to nerve growth factor, with no change in steady-state levels of Kv2.1 mRNA (43). It has been proposed that phosphorylation mechanisms, known to affect protein synthesis at the initiation or elongation phase (44), could be responsible. It is possible that changes in channel protein synthesis or degradation might be also responsible for the decreased Kv1.3 levels in T cells during hypoxia.

Similar to pulmonary artery smooth muscle cells, prolonged hypoxia does not alter the expression of auxiliary Kvβ subunits in T cells. Kv1.3 channels in T lymphocytes exist in association with Kvβ2 subunits (45). These subunits promote Kv channel surface expression and stabilize the channel complexes (33). It has been reported that during T cell mitogenesis, Kvβ2 gene expression is enhanced and this increase might account for the increase in functional Kv1.3 channels (33). Therefore, it is possible that a decrease in expression of Kvβ subunits could contribute to the observed decrease in functional Kv1.3 channels during hypoxia. Moreover, the Kvβ subunits are of particular interest in hypoxia studies because they have been proposed as possible O₂ sensors due to their similarity to NADPH-oxido-reductase enzymes (46–48). Our results indicate that Kvβ2 subunit expression is not regulated by hypoxia, thus suggesting that the expression of Kv1.3 channels during hypoxia is independent of the Kvβ2 subunit levels.

Although both acute and chronic effects of hypoxia on K⁺ channels have been reported, the mechanisms underlying these responses are not fully understood. Furthermore, it is not clear if these two effects are directly correlated or if they occur independently of each other. Our results indicate that a decrease in Kv1.3 levels similar to that observed in hypoxia is produced by exposing the cells in normoxia to a blocker of Kv channels (4-AP). Kv channel blockade, like hypoxia, decreases Kv1.3 protein levels but not mRNA. The down-regulation of the Kv1.3 α subunit is selective, as it is not observed for other K⁺ channels such as SK2 or other Kv channel subunits. This similarity between hypoxic and 4-AP-mediated responses suggests that the long-term consequences of hypoxia on Kv1.3 channel expression might be linked to the initial Kv1.3 channel blockade. It has been previously shown that 4-AP can mimic the acute hypoxic response in other cell types. Exposure to either 4-AP or hypoxia inhibits the Kₒ₂ current and induces membrane depolarization in pulmonary artery smooth muscle cells (36). It is possible that the membrane depolarization or other events triggered by the inhibition of the activity of Kv1.3 channels in T cells can ultimately regulate its protein expression. Expression of another member of the Kv1 family, Kv1.5, was shown to be down-regulated by membrane depolarization in pituitary cells (49).
Functional implications of Kv-2 channels in T cells

The ability of Kv1.3 channels to sense changes in O2 availability could have important implication in T cell function. Kv1.3 channels play an important role in lymphocyte activation by providing the driving force to maintain the influx of Ca2+ from the CRAC. A sustained increase in intracellular Ca2+ is necessary to drive many events in T cell activation, such as cytokine production and proliferation (2, 15, 50). It is well known that inhibition of Kv1.3 channels induces membrane depolarization and suppresses the activation response of human T cells (18, 31, 51, 52). It has been shown that hypoxia inhibits T cell proliferation and affects cytokine release (1, 5–8, 10, 53). Our data support the notion that hypoxia inhibits T cell proliferation. Furthermore, we showed that the effect of hypoxia involves membrane-delimited mechanisms as it was only observed when the mitogenic stimuli used involved the TCR complex and thus activation of the CRAC channels. Proliferation induced by stimuli that bypassed the membrane was not affected by hypoxia. This is expected if the decrease in proliferation during hypoxia is mediated by the hypoxic inhibition of the Kv1.3 channels. Still, the functional consequences of hypoxia may be different depending on the T cell activation state. In fact, Kv1.3 channels constitute the dominant conductance in resting and chronically activated T cells while K(Ca) channels are instead important in controlling T cell activation in acutely activated cells (31, 51, 52).

In conclusion, our data indicate that the Kv1.3 channels expressed in T lymphocytes are O2 sensitive. Inhibition of Kv1.3 channel expression and function by hypoxia could explain the changes in cellular proliferation that occurs in T cells exposed to hypoxia.

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References


