Hypoxia modulates early events in T cell receptor-mediated activation in human T lymphocytes via Kv1.3 channels

Jennifer R. Robbins[1,4], Susan Molleran Lee[3], Alexandra H. Filipovich[3], Peter Szigligeti[1], Lisa Neumeier[1], Milan Petrovic[1] and Laura Conforti[1,2]

[1]*Department of Internal Medicine and* [2]*Department of Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, OH 45267, USA*
[3]*Division of Hematology/Oncology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45267, USA*
[4]*Department of Biology, Xavier University, Cincinnati, OH 45207, USA*

T lymphocytes are exposed to hypoxia during their development and when they migrate to hypoxic pathological sites. Although it has been shown that hypoxia inhibits Kv1.3 channels and proliferation in human T cells, the mechanisms by which hypoxia regulates T cell activation are not fully understood. Herein we test the hypothesis that hypoxic inhibition of Kv1.3 channels induces membrane depolarization, thus modulating the increase in cytoplasmic Ca^{2+} that occurs during activation. Hypoxia causes membrane depolarization in human $CD3^+$ T cells, as measured by fluorescence-activated cell sorting (FACS) with the voltage-sensitive dye $DiBAC_4(3)$. Similar depolarization is produced by the selective Kv1.3 channel blockers ShK-Dap^{22} and margatoxin. Furthermore, pre-exposure to such blockers prevents any further depolarization by hypoxia. Since membrane depolarization is unfavourable to the influx of Ca^{2+} through the CRAC channels (necessary to drive many events in T cell activation such as cytokine production and proliferation), the effect of hypoxia on T cell receptor-mediated increase in cytoplasmic Ca^{2+} was determined using fura-2. Hypoxia depresses the increase in Ca^{2+} induced by anti-CD3/CD28 antibodies in ∼50% of lymphocytes. In the remaining cells, hypoxia either did not elicit any change or produced a small increase in cytoplasmic Ca^{2+}. Similar effects were observed in resting and pre-activated $CD3^+$ cells and were mimicked by ShK-Dap^{22}. These effects appear to be mediated solely by Kv1.3 channels, as we find no influence of hypoxia on IKCa1 and CRAC channels. Our findings indicate that hypoxia modulates Ca^{2+} homeostasis in T cells via Kv1.3 channel inhibition and membrane depolarization.

(Resubmitted 21 December 2004; accepted 24 January 2005; first published online 27 January 2005)
Corresponding author L. Conforti: Department of Internal Medicine, 231 Albert Sabin Way, University of Cincinnati, Cincinnati, OH 45267-0585, USA. Email: laura.conforti@uc.edu

Hypoxia (a decrease in O_2 availability) can occur in physiological as well as pathological conditions. Hypoxic areas have been measured in lymph nodes and spleen as well as in wounds, solid tumours, atheromatous plaques, bone fractures and joints with rheumatoid arthritis (Lewis *et al.* 1999; Brown, 2000; Caldwell *et al.* 2001; Hockel & Vaupel, 2001). Although hypoxia has been associated with poor prognosis in solid tumours and has been shown to retard wound healing, the basic mechanisms by which the different cells at the hypoxic site respond to the change in O_2 availability are still poorly understood (Lewis *et al.* 1999; Hockel & Vaupel, 2001). T cells are exposed to hypoxia when homing in on the lymphoid organs as well as when they reach pathological sites where they are expected to establish an appropriate defense. Thus it is possible that both T cell differentiation and function can be affected by hypoxia. Indeed, it has been proposed that the decreased immune surveillance previously observed in solid tumours could be explained in part by the effects of the tumour microenvironment on responding immune cells (Whiteside, 1998; Marincola *et al.* 2000).

Already, numerous studies have indicated that hypoxia inhibits proliferation and regulates cytokine production and release in T cells (Loeffler *et al.* 1990, 1991; Loeffler *et al.* 1992; Zuckerberg *et al.* 1994; Naldini & Carraro, 1999; Caldwell *et al.* 2001; Lukashev *et al.* 2001; Conforti *et al.* 2003; Makino *et al.* 2003); however, controversy still exists regarding the effect of hypoxia on T cell cytotoxicity (Loeffler *et al.* 1990; Caldwell *et al.* 2001), and the mechanism by which hypoxia exerts its effects has not been fully elucidated. In this study we have investigated the effect of hypoxia on T cell receptor (TCR)-mediated activation in human T lymphocytes. T cell activation results from the concerted function of

 DOI: 10.1113/jphysiol.2004.081893

various membrane channels and signalling molecules (Lewis, 2001). Engagement of antigen by the TCR activates phospholipase C and induces the release of Ca^{2+} from intracellular stores, which causes the calcium release activated calcium (CRAC) channels to open. The sustained influx of calcium through CRAC channels is essential to activate T cells, regulating both proliferation and cytokine production. Potassium channels provide a balanced cation efflux to maintain the necessary electrochemical driving force for Ca^{2+} influx. The voltage-dependent K^+ (Kv) channel encoded by the *Kv1.3* gene (named Kv1.3 channel according to the IUPHAR nomenclature, or, according to other nomenclatures, MK3, RCK3, Kv3 and n channel) and the Ca^{2+}-activated K^+ (K_{Ca}) channel encoded by h*IKCa1* (IKCa1; KCa3.1 according to the IUPHAR nomenclature, also named SK4 and KCNN4) are the major determinants of membrane potential in T cells (Lewis & Cahalan, 1995; Cahalan *et al.* 2001). These channels are present in variable amounts on the T cell surface, depending on the cell's differentiation state, and are differentially regulated by the membrane potential (Kv1.3) and intracellular Ca^{2+} (IKCa1) (Cahalan *et al.* 2001). We have recently shown that hypoxia inhibits both Kv1.3 channel expression and function in human T cells and that this inhibition correlates with a decrease in TCR-mediated proliferation (Conforti *et al.* 2003). This indicates that Kv1.3 channels play an additional role in Ca^{2+} maintenance by acting as part of the T cell oxygen-sensing apparatus. Similar channels are also found in chemosensitive cells such as carotid body type I cells and pulmonary artery smooth muscle cells, and modulation of these channels by hypoxia is a very early event in the overall process of oxygen sensing (Lopez-Barneo *et al.* 2001).

Here, we investigate the acute effects of hypoxia on several steps of activation in individual T cells. We find that hypoxia-induced inhibition of Kv1.3 current causes depolarization in $CD3^+$ T cells, thus decreasing the driving force for calcium influx into cells. We also show that hypoxia depresses the cytoplasmic calcium levels achieved after TCR stimulation. Interestingly, cell-by-cell analysis shows a variability in the response to hypoxia among human $CD3^+$ cells.

Methods

Cells

Peripheral blood mononuclear cells (PBMC) were obtained from consenting healthy adult donors and prepared by Ficoll density gradient (ICN Biomedicals, Aurora, OH, USA) as previously described (Conforti *et al.* 2003). T cells were separated from PBMCs using the E-rosette technique (StemCell Tech., Vancouver, Canada). FACS analysis showed that 98% of the cells isolated by E-rosetting were $CD3^+$, $58 \pm 2\%$ $CD4^+$ and $39 \pm 2\%$ $CD8^+$ ($n = 5$). T lymphocytes were maintained in RPMI medium supplemented with 10% pooled male human AB serum (Intergen, Milford, MA, USA), 200 units ml^{-1} penicillin, 200 $\mu g\,ml^{-1}$ streptomycin, 1 mM Hepes and maintained in a humidified incubator at 37°C with 5% CO_2. $CD3^+$ T cells were maintained in culture either alone or in the presence of autologous PBMCs.

Membrane potential measurements by flow cytometry

Purified human T cells were suspended (*ca* 1×10^6 ml^{-1}) in a solution of the following composition (mM): 150 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 glucose (pH 7.4). T cells were then exposed to one or more of the following conditions for 15 min to 2 h at room temperature: 10 mM TEA; 10 nM margatoxin; 10 nM ShK-Dap^{22}; increasing concentrations of K^+ (25, 50 and 75 mM) and hypoxia. To study the effect of hypoxia on membrane potential, cells were maintained in a modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA) in an atmosphere saturated with 1% O_2, 5% CO_2 and 94% N_2. Graded potassium solutions were made by altering the KCl and NaCl in the solution: KCl concentration was set to 5 (normal), 25, 50 and 75 mM whereas the NaCl concentration was adjusted to a total monovalent cation concentration of 155 mM. To measure the membrane potential, 300 nM *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) dye (Molecular Probes, Eugene, OR, USA) was added to the cells, and 5 min later the fluorescence intensity of the T cell population was analysed with a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA); 488 nm wavelength excitation. $DiBAC_4(3)$ was prepared in DMSO according to the manufacturer's instructions. All flow cytometric analyses were accomplished using CellQuest software (Becton-Dickinson).

Measurement of cytoplasmic Ca^{2+}

Cells on poly L-lysine (Sigma)-coated glass coverslips were loaded in 1 μM fura-2 AM (Molecular Probes) for 30–35 min at 22–24°C in RPMI, rinsed with 0.5 mM Ca^{2+} Ringer solution (mM: 155 NaCl, 4.5 KCl, 2.5 $MgCl_2$, 10 Hepes, 10 glucose, 0.5 $CaCl_2$, pH 7.4) and stored in the dark 10–90 min before use. Fura-2 intensity was measured on a Cyt-Im2 Ca^{2+} imaging system (Intracellular Imaging, Cincinnati, OH, USA) using the ratiometric method of Grynkiewicz *et al.* (1985). Cells were imaged on a Nikon inverted epifluorescence microscope equipped with a 20 × superfluor objective, NA 0.75, and the dye was excited alternately with 340 and 380 nm light from a xenon arc lamp. Light emitted from the dye was passed through a 535 WB35 emission filter and intensity values averaged over 0.5–0.7 s intervals for analysis. For activation with

Dynabeads CD3/CD28 (Dynal Biotech, Lake Success, NY, USA), cells were recorded while bathed in 1 ml 0.5 mM Ca^{2+} Ringer solution for 2–3 min, then 30 μl Dynabeads were pipetted into the bath. After 3–4 min, the bath was perfused with 0.5 mM Ca^{2+} Ringer solution for 1–2 min to wash away unattached beads. Visual inspection revealed that beads attached to cells remained throughout the perfusion that followed. Cells were then perfused with hypoxic 0.5 mM Ca^{2+} Ringer solution, and after 4–5 min of observation, 2–4 μM ionomycin was added as a positive control.

Exposure of cells to hypoxia

During Ca^{2+} imaging and electrophysiological experiments, the effect of hypoxia was studied by switching from a perfusion medium bubbled with air (21% O_2, 150 mmHg) to a medium equilibrated with 100% N_2 (P_{O_2} in the perfusion chamber *ca* 20 mmHg) (Conforti *et al.* 2003). The P_{O_2} in the perfusion chamber was measured by a polarographic oxygen electrode (WPI, Sarasota, FL, USA). To study the effect of hypoxia on membrane potential by FACS, cells were maintained in a modular incubator chamber (Billups-Rothenberg, Inc.), in an atmosphere saturated with 1% O_2 (*ca* 8 mmHg) for 15 min to 2 h prior to performing flow cytometric analysis. Immediately upon removal from the hypoxic chamber, the cells were stained with $DiBAC_4(3)$ for 5 min at room atmosphere and temperature and then analysed. Data acquisition time was approximately 30–60 s per tube, also at room atmosphere. In a subset of experiments cells were returned to the hypoxic chamber after addition of $DiBAC_4(3)$. Similar results were obtained in the two sets of experiments, indicating that a prolonged reoxygenation is necessary for the membrane potential to return to more hyperpolarized voltages (as confirmed by current clamp data shown in Fig. 1*C*). Furthermore, in each set of experiments, normoxia, hypoxia and 75 K^+ samples were run in parallel, and the shift in fluorescence by hypoxia and 75 K^+ were always relative to normoxia.

Electrophysiology

K_{Ca} currents (IKCa1) were recorded in whole-cell patch configuration in pre-activated (blast) T cells exposed to 4 μg ml^{-1} phytohemmaglutinin (PHA) for more than 48 h. The external solution for activating and recording IKCa1 currents had the following composition (mM): 160 NaCl, 4.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, and 10 Hepes, pH 7.4. The pipette solution was composed of (mM): 145 potassium aspartate, 8.5 $CaCl_2$, 10 K_2EGTA, 2.0 $MgCl_2$, and 10 Hepes, pH 7.2, with an estimated free [Ca^{2+}] of 1 μM (Ghanshani *et al.* 2000). IKCa1 was measured in voltage-clamp mode by ramp pulse depolarization from −120 mV to +40 mV (200 ms duration, every 30 s, −80 mV holding potential). Data were corrected for a liquid junction potential of −10 mV (−9.9 ± 0.6, $n = 5$). IKCa1 slope conductance was measured between −100 mV and −60 mV, to avoid contamination by the Kv current. Mitogen pre-activated T cells are characterized by a cell capacitance higher than resting T cells and increased number of IKCa1 channels (Ghanshani *et al.* 2000; Fanger *et al.* 2001). Mitogen pre-activated $CD3^+$ cells had a membrane capacitance significantly higher than resting T cells: 5.1 ± 0.3 pF ($n = 26$) and 1.4 ± 0.2 pF ($n = 17$; $P < 0.001$), respectively. Similar capacitance values have been reported for resting and activated human T cells (Wulff *et al.* 2003*b*). We estimated the number of IKCa1 channels per mitogen pre-activated T cell by dividing the IKCa1 conductance for the IKCa1 single channel conductance (11 pS) and found it to be 248 ± 34 channels per cell ($n = 27$).

CRAC currents were recorded in freshly isolated resting T cells in whole cell configuration. The pipette and bath solutions had the following compositions (mM): 135 caesium aspartate, 12 BAPTA, 1 calcium aspartate, 4 MgATP, 10 Hepes (pH 7.4) and 110 $NaMeSO_3$, 20 $Ca(MeSO_3)_2$, 10 glucose, 10 Hepes (pH:7.4), respectively. Bath and pipette solutions had equal osmolarity, between 300 and 305 mosmol l^{-1}. CRAC currents were elicited by ramp depolarization from −120 mV to +40 mV (−20 mV holding potential) every 10 s. The recording protocol was corrected by −10 mV to account for the liquid junction potential. Leak currents were measured upon breaking into the whole-cell configuration, before induction of CRAC currents by Ca^{2+} store depletion, and subtracted.

Membrane potential was measured in whole-cell configuration in current-clamp mode as previously described (Zhu *et al.* 1996). The external solution had the following composition (mM): 150 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, and 10 Hepes, 10 glucose, pH 7.4. The pipette solution was composed of (mM): 134 KCl, 1.0 $CaCl_2$, 10 EGTA, 2.0 $MgCl_2$, and 10 Hepes, 5 Na-ATP, pH 7.4,

Experiments were performed using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). The digitized signals were stored and analysed using pCLAMP 8 software (Axon Instruments).

Data analysis

All data are presented as means ± S.E.M., unless otherwise indicated. Statistical analyses were performed using Student's *t* test (paired or unpaired); $P \leq 0.05$ was defined as significant.

Calcium data analysis

For all calcium ratiometric image analysis, Excel software with Visual BASIC scripting (Microsoft) and a Macintosh

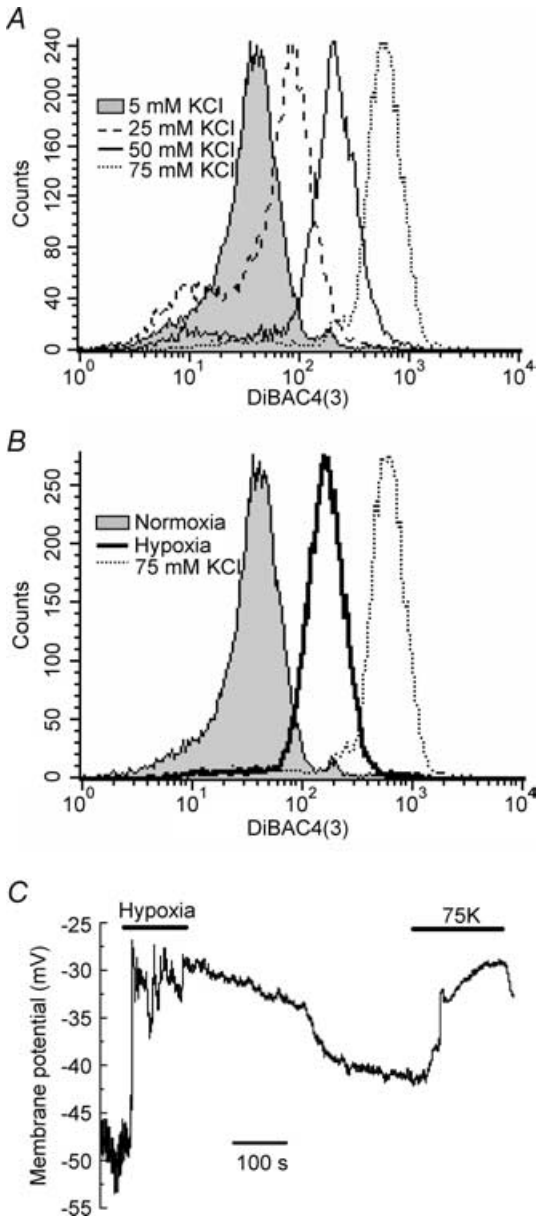


Figure 1. Hypoxia induces membrane depolarization in human CD3$^+$ lymphocytes
A, hypoxia-induced membrane depolarization is indicated by the shift in DiBAC$_4$(3) fluorescence in the same direction as that induced by progressively higher K^+ concentrations. Graded potassium solutions were made by altering the KCl and NaCl in the solution (see Methods). For graded K^+ solutions, the KCl concentration was set to 5 (normal), 25, 50 and 75 mM whereas the NaCl concentration was adjusted to a total monovalent cation concentration of 155 mM. *B*, exposure to hypoxia for 15 min caused depolarization. These data are representative of 4 separate experiments in 4 donors. *C*, effect of hypoxia on membrane potential as determined in resting T lymphocytes by current clamp. Membrane potential was recorded continuously in normoxia and while switching to hypoxia (20 mmHg) or 75 mM KCl. The time of exposure to either hypoxia or 75 KCl is indicated by the bars above the trace.

G4766 computer was used. Due to the variability inherent in the Dynabead and hypoxia responses, all cell traces were analysed individually. Each 340/380 intensity ratio was calculated and the trace then smoothed by averaging each ratio time point with each of its immediate neighbours (thus, each point in Figs 4 and 7 represents ∼1.5–2 s). Cells that exhibited a sudden, sustained rise in calcium 1–3 min after addition of Dynabeads were defined as having responded to a bead. In some cells, such activation was transient (not sustained) and cells rapidly returned to baseline without environmental provocation. These traces were not included in Fig. 5 analysis, as the effect of hypoxia could not be isolated. We also excluded traces with unsteady (sloping) baseline calcium (presumably due to fura-2 leakage or physiological processes unrelated to the experiment). Of the remaining ∼74% of cells, 47% contacted and responded to beads. Response to hypoxia was calculated as the difference between the average of 10 smoothed 340/380 ratios before addition of ionomycin (after hypoxia) and the average of 10 smoothed 340/380 ratios immediately preceding the introduction of hypoxia. Calcium response to beads (Fig. 6) was calculated in the same way, but the average of the last 10 points before the addition of beads was subtracted from the average of the last 10 points before introduction of hypoxia. Baseline calcium values (Fig. 6) were calculated as the average of all 340/380 ratio values before addition of Dynabeads, normalized to the average baseline ratio of that day's experiments to account for differences in fura-2 loading, differential effects of lamp intensity levels on bound and unbound fura-2, and possible daily fluctuations in $[Ca^{2+}]$ in solution.

Chemicals and solutions

PHA, tetraethylammonium chloride (TEA), 2-aminoethyl diphenyl borate (2-APB) and ionomycin were obtained from Sigma. Margatoxin and ShK-Dap^{22} were purchased from Alomone Laboratories (Jerusalem, Israel) and BACHEM Bioscience Inc. (King of Prussia, PA, USA), respectively. Clotrimazole (Calbiochem, San Diego, CA, USA) was dissolved in DMSO and used at a final 1 : 10 000 dilution. Dynabeads CD3/CD28 T Cell Expander were obtained from Dynal Biotech Inc.

Results

Hypoxia induces membrane depolarization in human CD3$^+$ cells

It has been previously established that hypoxia inhibits Kv1.3 channels in human T cells (Conforti *et al.* 2003). Kv1.3 channels set the resting membrane potential of resting T cells and are required for normal lymphocyte activation both *in vivo* and *in vitro* (Leonard *et al.*

1992; Lewis & Cahalan, 1995). Inhibition of Kv1.3 channels by K^+ channel toxins induces membrane depolarization and suppresses the activation response of human T cells (Leonard *et al.* 1992; Koo *et al.* 1997). It is therefore possible that hypoxia induces depolarization in human T lymphocytes. In order to investigate this, we used the plasma membrane potential indicator dye $DiBAC_4(3)$. This is a fluorescent anionic bis-oxonol dye that is normally excluded from the cytoplasm by the negative resting potential of the plasma membrane, but depolarization allows dye entry, increasing intracellular fluorescence. The use of $DiBAC_4(3)$ in combination with fluorescence-activated cell sorting (FACS) permitted us to observe the progressive depolarization of $CD3^+$ cells isolated from human peripheral blood as they were exposed to graded concentrations of extracellular K^+ ($[K^+]_o$; Fig. 1*A*).

To test whether changes in membrane potential occur in hypoxia, we exposed T cells to 1% O_2 for 15 min. Hypoxia did cause membrane depolarization, as indicated by a shift in fluorescence similar to that observed in high $[K^+]_o$ (Fig. 1*B*). The effect of hypoxia remained statistically significant even after 2 h exposure to hypoxia (Fig. 2*A*). The relative mean channel fluorescence (MCF) with respect to normoxia after 15 min and 2 h in hypoxia was 2.4 ± 0.6 ($n = 4$ independent experiments from 4 donors) and 1.4 ± 0.1 ($n = 3$ donors), respectively ($P = 0.2$). This depolarization exclusively occurs at the plasma membrane since $DiBAC_4(3)$ is a plasma membrane potential specific dye that does not sense changes in mitochondrial potential (Bortner *et al.* 2001).

The depolarizing effect of hypoxia was confirmed in current-clamp experiments (Fig. 1*C*). Hypoxia (20 mmHg) induced a 15.5 ± 2.3 mV depolarization from -52.0 ± 3.2 mV to -36.5 ± 4.7 mV ($n = 4$) in resting CD3 lymphocytes. Similar resting membrane potentials were reported for T cells ((Panyi *et al.* 2004*b*). The hypoxia-induced depolarization developed immediately upon switching to an hypoxic perfusate, and it was in part reversible, although a longer time was necessary to return to more hyperpolarized voltages.

Membrane depolarization during hypoxia is mediated by Kv1.3 channel block

If membrane depolarization in hypoxia results from the hypoxic inhibition of Kv1.3 channels, as hypothesized, one would expect that blockers of Kv1.3 currents should mimic the effect of hypoxia. Indeed, exposure to tetraethylammonium chloride (TEA, 10 mM), or to specific Kv1.3 blockers including margatoxin (MgTX, 10 nM) (Leonard *et al.* 1992) and ShK-Dap^{22} (10 nM) (Kalman *et al.* 1998), induced depolarization similar to hypoxia (Fig. 2). Furthermore, pre-exposure to ShK-Dap^{22} prevented further depolarization by hypoxia (Fig. 3), indicating that the two effects on membrane potential are not additive. We found no significant difference between the effects of ShK-Dap^{22} and/or hypoxia (Fig. 3*B*).

Unexpectedly, we also found that a subset of cells in 5 out of 8 donors were less responsive or non-responsive to both hypoxia and ShK-Dap^{22}, as evidenced by the leftward 'shoulder' of the $DiBAC_4(3)$ fluorescence peak under these

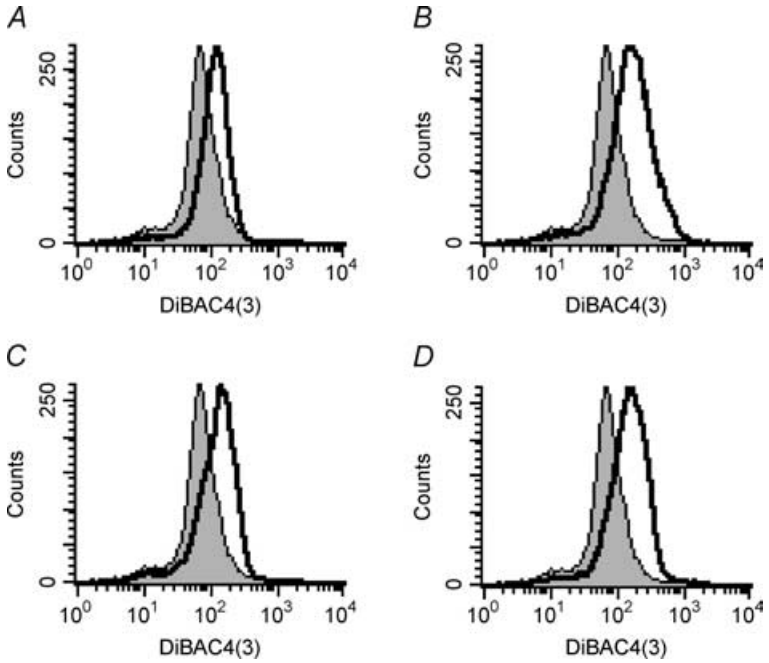


Figure 2. Hypoxia inhibits the ShK-Dap^{22}-sensitive Kv1.3 current in human T cells
Cells were exposed to: *A*, hypoxia; *B*, 10 nM MgTX; *C*, 10 mM TEA; or *D*, 10 nM ShK-Dap^{22} for 120 min, followed by measurement of $DiBAC_4(3)$ fluorescence. An increase in fluorescence intensity corresponding to membrane depolarization was observed in all cases. These data are representative of 2 experiments in 2 donors. Normoxia is indicated by the shaded region, while continuous lines indicate the Kv1.3 channel blocker.

conditions (as in Fig. 3*A*). This population varied in size depending on the donor and day tested, and suggests some heterogeneity among $CD3^+$ cell responses to hypoxia.

Hypoxia inhibits Ca^{2+} signal in resting and pre-activated T cells after TCR engagement

If hypoxia causes the membrane potential to rise, one would expect that it should therefore decrease the driving force for Ca^{2+} influx after TCR engagement. In order to explore the physiological consequences of hypoxia-induced depolarization, we stimulated cells with 4.5 μm diameter beads coated with a mixture of human anti-CD3 and anti-CD28 antibodies (Spisek *et al.* 2002), and then exposed them to hypoxia. This stimulus closely mimics the antigen presentation and subsequent T cell activation that occurs *in vivo*. We recorded calcium flux in 175–420 cells in each of five donors, stimulating both resting and mitogen (PHA) pre-activated $CD3^+$ cells either in isolation or co-cultured with PBMCs (Fig. 4; Table 1). Hypoxia depressed the calcium plateau in about half of stimulated cells (Fig. 4*A* and *E*; Table 1), but had little to no effect on unstimulated cells (Fig. 4*D*). Still, these latter cells respond to ionomycin (data not shown). The average increase in the 340/380 ratio due to ionomycin is 133 ± 4% (709 cells, from all behaviours). More cells showed plateau depression in pre-activated than in resting cells (Fig. 4*E*).

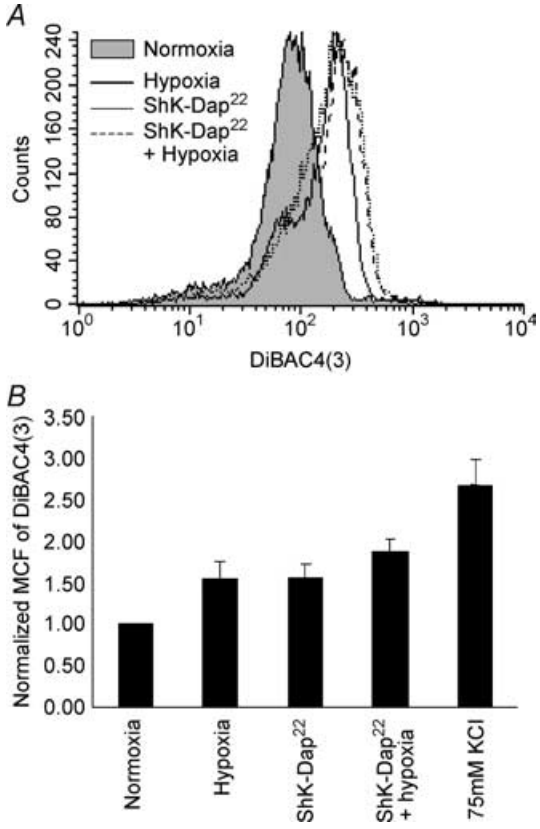


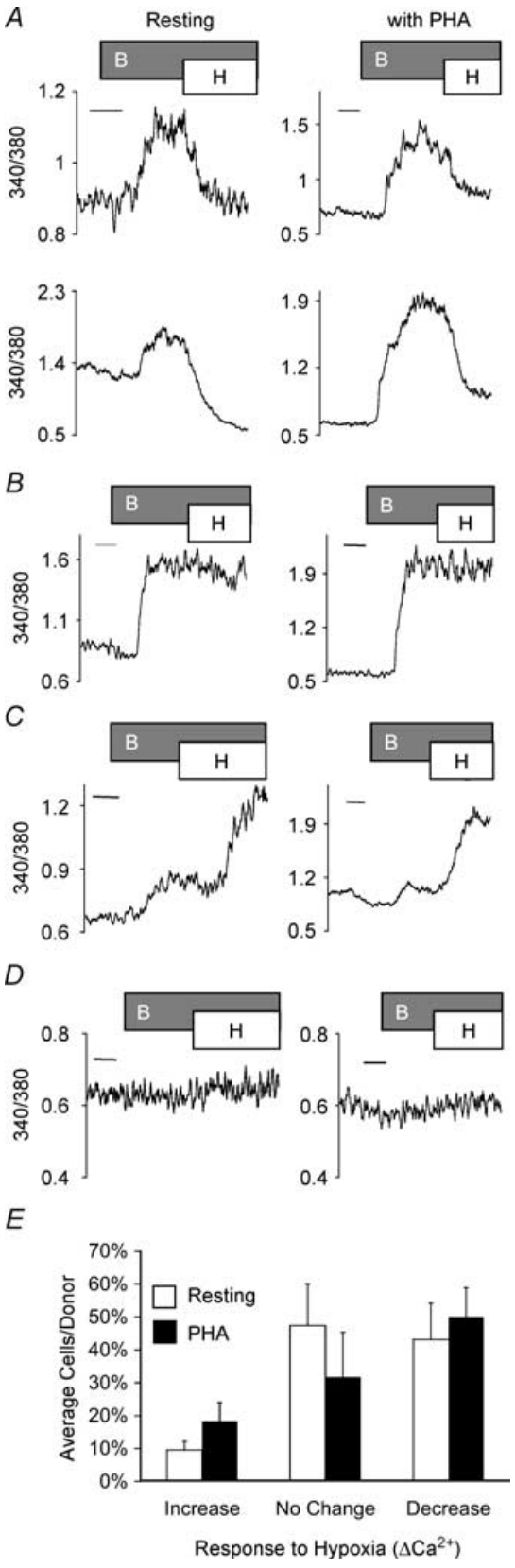
Figure 3. Effects of hypoxia and ShK-Dap^{22} are not additive
Cells were exposed to either hypoxia for 15 min or 10 nM ShK-Dap^{22} for 20 min, or ShK-Dap^{22} for 20 min, with the latter 15 min spent in hypoxia (ShK-Dap^{22} + hypoxia). *A*, a representative experiment showing $DiBAC_4(3)$ fluorescence (increased fluorescence corresponds to increased membrane depolarization). *B*, mean channel fluorescence (MCF) averaged over 6 trials and compared with normoxia. The data are the average of 6 separate donors except for 75 mM KCl ($n = 2$). The effect of hypoxia and ShK-Dap^{22} together was statistically indistinguishable from either individually ($P = 0.9$ hypoxia *versus* ShK-Dap^{22}; $P = 0.3$ hypoxia *versus* ShK-Dap^{22} + hypoxia; $P = 0.2$ ShK-Dap^{22} *versus* ShK-Dap^{22} + hypoxia).

A large but highly variable fraction of stimulated cells did not respond to hypoxia (Fig. 4*B* and *E*), while a small fraction actually increased cytoplasmic calcium after exposure to hypoxia (Fig. 4*C* and *E*). No statistically significant differences between isolated $CD3^+$ cells and those co-cultured with PBMCs were apparent ($P > 0.14$). Like other investigators (Hess *et al.* 1993), we found that some cells exhibited only a transient increase in calcium in response to stimulation that declined independently of hypoxia (Table 1); these were excluded from analysis since we could not isolate the effect of hypoxia. They may represent Th2 cells, which have previously been shown to clear intracellular Ca^{2+} more rapidly than their Th1 counterparts (Fanger *et al.* 2000).

That T cells respond differentially to hypoxia is perhaps not surprising, since so many functional T cell subsets exist (Swain, 2003). We analysed all the cells' individual responses to hypoxia and plotted them as a histogram (Fig. 5), which clearly shows the variability inherent in the hypoxia response. Mitogen pre-activated cells exhibited a slightly and insignificantly broader range of responses than did resting cells (Fig. 5*A*; $P = 0.4$). Multiple frequency peaks can be observed, suggesting multiple possible responses or degrees of response; by comparison, unstimulated cells (cells that did not respond to anti-CD3/CD28-coated beads) in the same experiments show consistently minimal responses to hypoxia (Fig. 5*B*).

Cytoplasmic calcium response to hypoxia correlates with baseline calcium levels as well as degree of response to stimulus

As previous studies have shown that calcium homeostasis is variably maintained depending on T cell subtype (Hess *et al.* 1993; Fanger *et al.* 2000), we analysed these features according to individual cell response to hypoxia (Fig. 6). The metrics we used are shown in a representative trace in Fig. 6*A*. We found that cells whose calcium levels decreased in response to hypoxia showed a slightly – but significantly – higher baseline (pre-stimulus) cytoplasmic calcium level (Fig. 6*B*; $P < 0.004$). Their baselines were more similar to those of unstimulated cells or cells that were stimulated only transiently than to those of cells



whose calcium levels did not change or even increased in hypoxia ($P > 0.09$). The level of response to stimulus also varied, with cells whose calcium levels decreased showing a significantly higher response to anti-CD3/CD28 beads, one that was more similar to that of transiently stimulated cells than to cells unaffected by hypoxia or whose calcium levels increased (Fig. 6*B*; $P < 0.03$). Finally, analysis of the rates of change in cytoplasmic calcium concentration in response to hypoxia showed that the small population of cells whose calcium levels increase did so significantly faster than cells exhibiting cytoplasmic calcium decline in response to hypoxia (Fig. 6*B*; $P < 0.01$).

Hypoxia effect on TCR-mediated activation is mediated by Kv1.3 channels

We found no significant difference in the response elicited by hypoxia as compared with that caused by the Kv1.3-specific blocker ShK-Dap^{22} (10 nM) (Fig. 7; $P = 0.27$). Here, mitogen pre-activated cells were stimulated by anti-CD3/CD28 followed by either the toxin or hypoxia; both were able to cause a similar distribution of responses. This strongly supports the conclusion that Kv1.3 is responsible for the effect we see on cells in hypoxia; however, we wished to rule out the possibility of contributions by other channels.

To confirm that hypoxia has no effect on the major port of Ca^{2+} influx, CRAC channels, we recorded CRAC currents in $CD3^+$ cells. CRAC currents were induced by depletion of intracellular Ca^{2+} stores. They displayed inward rectification and were blocked by high concentrations of 2-APB (Prakriya & Lewis, 2001; Schindl *et al.* 2002). We found that hypoxia has no effect on the Ca^{2+} depletion-induced CRAC currents (I_{CRAC}) in fresh T cells: the CRAC current amplitude at −80 mV was

Figure 4. Hypoxia causes a decrease in cytoplasmic calcium after stimulation by anti-CD3/CD28 beads in a subpopulation of human T cells from five donors
Cells were stimulated by addition of beads ('B'); after ∼150 s, free beads were washed from the chamber by perfusion and soon after, cells were exposed to hypoxia ('H'). Each panel shows representative traces of cytoplasmic calcium in individual resting (left column) or mitogen pre-activated (right column) lymphocytes. Bars, 60 s. *A*, bead stimulation occurred at different times, but many cells exhibited a sharp decline in cytoplasmic calcium levels when exposed to hypoxia. *B*, other cells from the same donors did not respond to hypoxia. *C*, a small fraction of cells actually increased cytoplasmic calcium levels ∼30–100 s after exposure to hypoxia. *D*, unstimulated cells (cells not contacting or responding to anti-CD3/CD28 beads) in the same experiments showed little or no response to hypoxia. *E*, visual inspection of stimulated cell traces allows placement of cells into one of three categories. Hypoxia elicited a decrease in cytoplasmic calcium from a widely variable average of ∼40% of lymphocytes in resting cells, or ∼50% in mitogen pre-activated cells. A small fraction of cells actually increased cytoplasmic calcium in response to hypoxia. Data are the averages of 5 donors, each with ∼70 stimulated cells.

Table 1. Hypoxia elicits varying responses on cytosolic Ca^{2+} in T lymphocytes (mixed $CD3^+$ and $CD3^+$ co-cultured with PBMCs) in five donors

	Resting cells				
	Decrease (%)	No change (%)	Increase (%)	*n*	Transient (%)
Donor 1	22	70	8	74	6
Donor 2	38	44	17	81	8
Donor 3	11	75	14	44	4
Donor 4	75	21	4	91	3
Donor 5	70	26	4	23	13
Average	43	47	10	63	7
	PHA pre-activated cells				
Donor 1	96	0	4	48	30
Donor 3	29	40	31	78	12
Donor 4	47	43	10	147	15
Donor 5	60	26	14	100	17
Average	50	32	18	76	15

n, number of anti-CD3/CD28-stimulated cells (not including transient responders). Transient responders are reported as a percentage of all cells (stimulated and unstimulated). These cells did not sustain high Ca^{2+} levels when stimulated, but rapidly returned to baseline without experimental provocation. Hence, they were excluded from hypoxia analysis.

-21.7 ± 5.4 pA in normoxia and -20.7 ± 4.7 pA after 3 min in hypoxia ($n = 7$, $P = 0.45$) (Fig. 8). To investigate the possibility that the other major regulator of membrane potential in primary T cells, the K_{Ca} channel encoded by the h*IKCa1* gene is inhibited by hypoxia, IKCa1 currents were recorded in PHA-activated $CD3^+$ cells. IKCa1 currents were inhibited by clotrimazole, an inhibitor of IKCa1 (Ishii *et al.* 1997). In contrast, exposure to hypoxia for 2–5 min did not affect IKCa1: slope conductance in normoxia and hypoxia were 2.7 ± 0.7 nS and 2.6 ± 0.8 nS, respectively ($n = 5$, $P = 0.4$, Fig. 9). We instead observed a significant current inhibition at depolarized voltages, where Kv1.3 currents still contribute to the total K^+ current recorded: peak K^+ current at $+30$ mV was 1870.4 ± 284.1 pA in normoxia and 1638.4 ± 223.8 pA in hypoxia ($n = 5$, $P = 0.03$).

Discussion

T cells are exposed to different degrees of oxygen tension (P_{O_2}) as they migrate from the lymph nodes to the tissues, where, during an immune response, they risk exposure to hypoxia at pathological sites such as solid tumours, wounds and inflamed areas. Although the mechanisms of T cell adaptation to hypoxia have been at the centre of various investigations over the past few years (Caldwell *et al.* 2001; Kojima *et al.* 2003; Makino *et al.* 2003), the acute effects of hypoxia on the early mechanics of TCR-mediated activation are not yet understood. Here, we report that hypoxia modulates human T lymphocyte membrane potential and Ca^{2+} homeostasis through inhibition of Kv1.3 channels. To our knowledge, this is the first mechanistic description of the functional consequences that hypoxia has on the early phase of TCR activation in a physiological population of human T lymphocytes.

Hypoxia's effect on T cell membrane potential and cytoplasmic calcium levels

The early transient Ca^{2+} influx evoked by TCR antigen cross-linking is a key triggering signal for T cell activation and it is generated by the concerted action of various ion channels on the T cell membrane. This influx must be sustained for 60–120 min for IL-2 to be expressed and activation to become antigen independent (Lewis, 2001), so any factors able to modulate initial Ca^{2+} influx ultimately have important implications for T cell function. In human T cells, Kv1.3 and IKCa1 channels are important regulators of T cell function, as they both contribute to the membrane potential and provide the driving force for Ca^{2+} entry into the cell. Modulation of these channels has great influence over shaping the Ca^{2+} signal (Rader *et al.* 1996; Cahalan *et al.* 2001).

Here we show, for the first time, that hypoxia induces T cell depolarization via inhibition of Kv1.3 channels. This is in agreement with our previous report that hypoxia inhibits Kv1.3 currents in human T cells (Conforti *et al.* 2003) and with previous studies showing that Kv1.3 blockers induce membrane depolarization and suppress the activation response of human T cells (Cahalan *et al.* 2001). Interestingly, our data also indicate that, although

the majority of T cells depolarize in hypoxia, a subset of cells appears to be less responsive or non-responsive to both hypoxia and Kv1.3 blockade (Fig. 3*A*), suggesting some degree of heterogeneity among CD3$^+$ cells' response to hypoxia. This functional heterogeneity is even more evident in the Ca^{2+} imaging studies where a milder hypoxia was used and where it was possible to analyse the downstream consequences of depolarization in a large number of T cells from healthy individuals, one cell at a time.

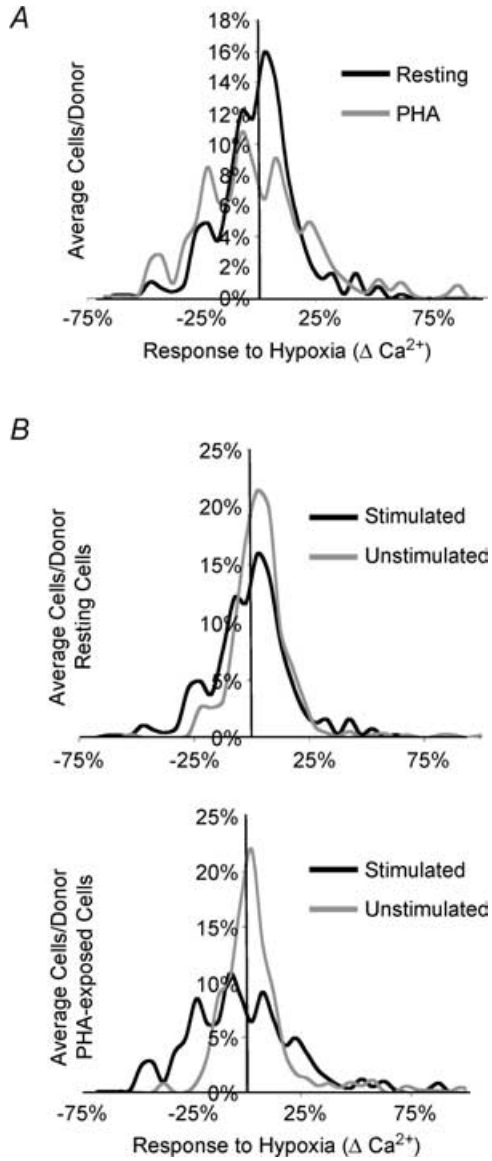


Figure 5. Response to hypoxia varies among T cells
This response is shown as the percentage change in 340/380 ratio due to hypoxia (calculated from ~10 s of averaged 340/380 ratios just before and approximately 4 min after onset of hypoxia). These experiments represent the averages of 5 donors with 650 stimulated cells (285 resting, 365 mitogen pre-activated) and 1733 unstimulated cells (766 resting and 967 mitogen pre-activated). *A*, distribution of responses to hypoxia suggests that change was graded over 3 or more subpopulations of cells, more evident in mitogen pre-activated cells than resting cells. *B*, unstimulated cells in the same experiments exhibited a single normal distribution of changes in calcium following hypoxia, while the response of stimulated cells was significantly more variable ($P < 0.01$) in both resting cells (top) and PHA pre-activated cells (bottom).

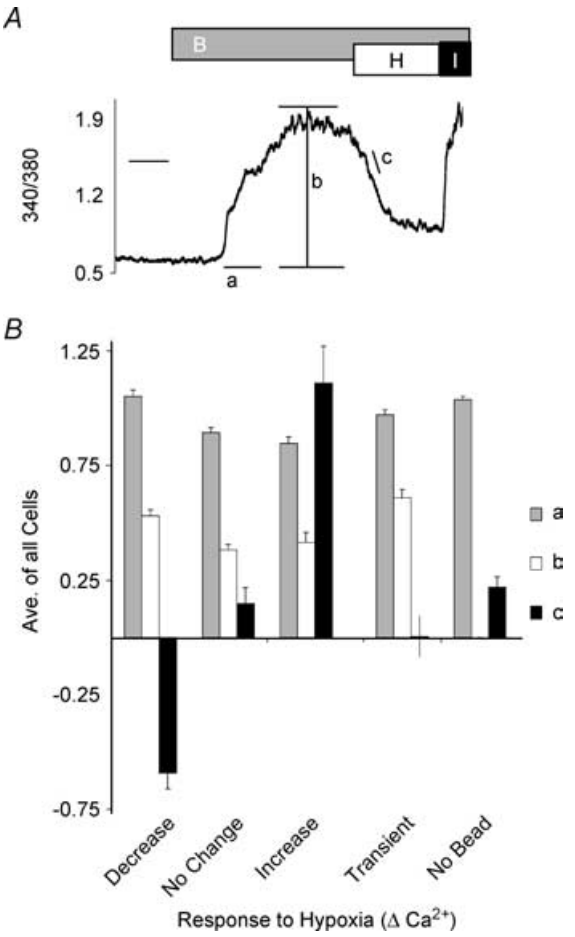


Figure 6. Comparison of calcium maintenance in the three T cell subpopulations
A, fluorescence intensities over time were analysed to find: (a) average 340/380 ratio baseline, normalized to the average baseline ratio of that day's experiments; (b) response to anti-CD3/CD28 bead stimulus (% change); and (c) highest magnitude slope over ~15 s ($\Delta 340/380\ s^{-1} \times 10^2$). Bar, 60 s. B, beads; H, hypoxia; black bar, ionomycin B. Cells whose cytoplasmic calcium decreased in hypoxia had slightly (but statistically significant) higher and more varied baseline 340/380 ratios, while cells whose calcium levels increased or did not change show no difference in calcium baseline. Cells that had either transient responses to beads or showed no effect of stimulus ('no bead') were not significantly different from each other. All 340/380 ratios were normalized to the average of the experiment. Cells whose cytoplasmic calcium decreased in hypoxia exhibited slightly (but significantly) higher responses to stimulus than those whose calcium levels increased or did not change. The calcium response to beads in these last two groups are statistically similar. Cells with transient responses to stimulus (responses that decline without changes in the environment) showed increases in calcium statistically similar to those of cells whose calcium levels decline in hypoxia. The maximum rate of cytoplasmic calcium change varied from cell to cell, with cells that increased cytoplasmic calcium levels in response to hypoxia changing fastest, on average. Cells that responded transiently to stimulus, or that did not respond at all show statistically similar maximum rates of change.

Our results clearly indicate a variability in the T cell response to hypoxia with the majority of the T cells (*ca* 50%) displaying a decrease in TCR-induced cytoplasmic Ca^{2+} concentration upon introduction of hypoxia. This can be explained by the inhibition of Kv1.3 currents during hypoxia. Indeed, effects similar to hypoxia were evoked by the Kv1.3 specific blocker ShK-Dap^{22} (Fanger *et al.* 2001), as shown in Fig. 7, although close examination of the distribution suggests that the hypoxia response may yield more degrees of response. This could indicate that channels other than Kv1.3 might be responding to hypoxia, or that signalling intermediates linking hypoxia to Kv1.3 channel activity might be differentially expressed in different T cells.

Electrophysiological studies presented herein argue against modulation of IKCa1 and CRAC channels by hypoxia. Hypoxia did not decrease Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) in fresh T cells. Whole-cell voltage-clamp experiments in pre-activated T cells also indicated that IKCa1 channels were not regulated by hypoxia. Still, we cannot exclude the possibility that, in different experimental conditions that better preserve the intracellular environment, an oxygen sensitivity might be displayed by these channels and thus partially contribute to the multiple and differential Ca^{2+} handling during hypoxia in T cell subsets. Furthermore, it remains possible that other channels expressed in T cells, such as L-type-related Ca^{2+} and TRPM4 channels might display O_2 sensitivity (Launay *et al.* 2004; Stokes *et al.* 2004).

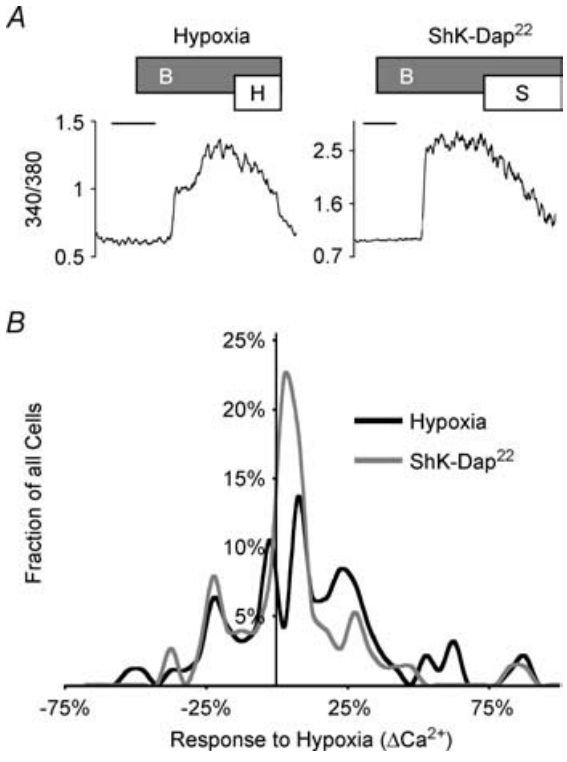


Figure 7. When substituted for hypoxia, the Kv1.3-specific blocker ShK-Dap^{22} has statistically similar effects on TCR-induced increase in cytoplasmic [Ca^{2+}]
A, representative traces from a single donor in which hypoxia ('H') or ShK-Dap^{22} ('S') induces a decline in the calcium plateau after stimulation with anti-CD3/CD28 beads ('B'). Bar, 60 s. *B*, distribution of hypoxia responses in mitogen pre-activated cells from 2 donors ($n > 75$ cells in each category).

Diversity of T cell responses to hypoxia

The variability of the hypoxic response in human T cells reflects the high heterogeneity of T cells in the body that has also been seen by others who have measured

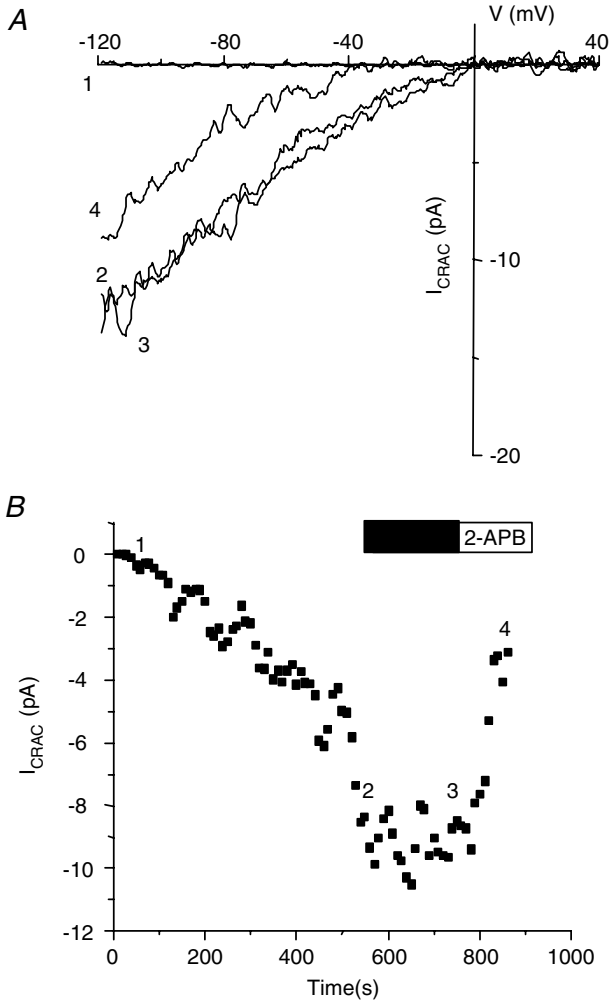


Figure 8. Hypoxia has no effect on CRAC currents in human T cells
A, representative current–voltage relationships were obtained from resting T cells by ramp depolarization as described in Methods. The current traces reported correspond to the time points indicated in the time-course graph below and were taken upon braking into whole-cell configuration (1), after Ca^{2+} store depletion and maximal activation of CRAC current in normoxia (2) and hypoxia (20 mmHg; 3), and finally after application of 50 μM 2-APB (4). *B*, time-dependent changes in CRAC current amplitude measured at −80 mV. Hypoxia elicits no response, but 2-APB, which in high concentrations blocks CRAC currents, causes current inhibition.

calcium responses on a cell-by-cell basis (Hess *et al.* 1993; Verheugen & Vijverberg, 1995; Guse, 1998). Initial experiments on-going in our laboratory on naïve and memory $CD4^+$ and $CD8^+$ cells have not conclusively demonstrated the exclusive association of membrane depolarization with hypoxia in any T cell subset defined by those surface markers. Further studies are needed to decipher the factors that account for the observed heterogeneity. Our finding that hypoxia-sensitive cells showed lower cytoplasmic calcium baseline levels and larger increases in response to stimulus than did hypoxia-insensitive cells suggests that hypoxia sensitivity might correlate with T cell activation state. This hypothesis is further supported by the slightly lower percentage of hypoxia-insensitive cells in PHA activated cells compared with resting T cells. It is well established that activation of various signalling molecules such as src protein tyrosine kinases (src-PTK) occurs upon engagement of the TCR complex (Palacios & Weiss, 2004). Recently, Cayabab *et al.* (2000) have shown that src PTKs mediate Kv1.3 channel response to anoxia and glucose deprivation in rat microglia. Therefore it is possible that the early signalling events triggered by TCR binding might influence the Kv1.3 channel sensitivity to hypoxia and consequently the overall Ca^{2+} response.

One might hypothesize that the different sensitivity to hypoxia might be due to individual cells' varied ratios of Kv1.3/IKCa1 channel expression, and it has been recently shown that the Kv1.3/IKCa1 ratio varies by T cell function and activation state (Wulff *et al.* 2003*a*,*b*). Resting $CD3^+$ naïve, memory (TCM) and effector (TEM) cells express more Kv1.3 channels than IKCa1. As they move from resting to proliferating blast cells, IKCa1 channels become predominant in naïve and TCM cells, while the number of Kv1.3 channels increases dramatically in TEM cells (Wulff *et al.* 2003*b*). Thus, it has been proposed that Kv1.3 channels control Ca^{2+} homeostasis in resting T cells and activated TEM, while IKCa1 channels are the main modulators of Ca^{2+} signalling and proliferation in mitogen pre-activated naïve and TCM cells (Wulff *et al.* 2003*a*). Based on this, we would have predicted that hypoxia has an overall inhibitory effect in resting cells, while a subpopulation distribution was expected for blast cells. This was not the case and similar trends (although more pronounced in pre-activated cells), were observed in both sets of cells. In explaining this, experimental differences between our work and that of others should be first considered. Most of the studies aimed at identifying the role of K^+ channel subtypes in Ca^{2+} handling use thapsigargin (TG) as an initial Ca^{2+}-triggering stimulus

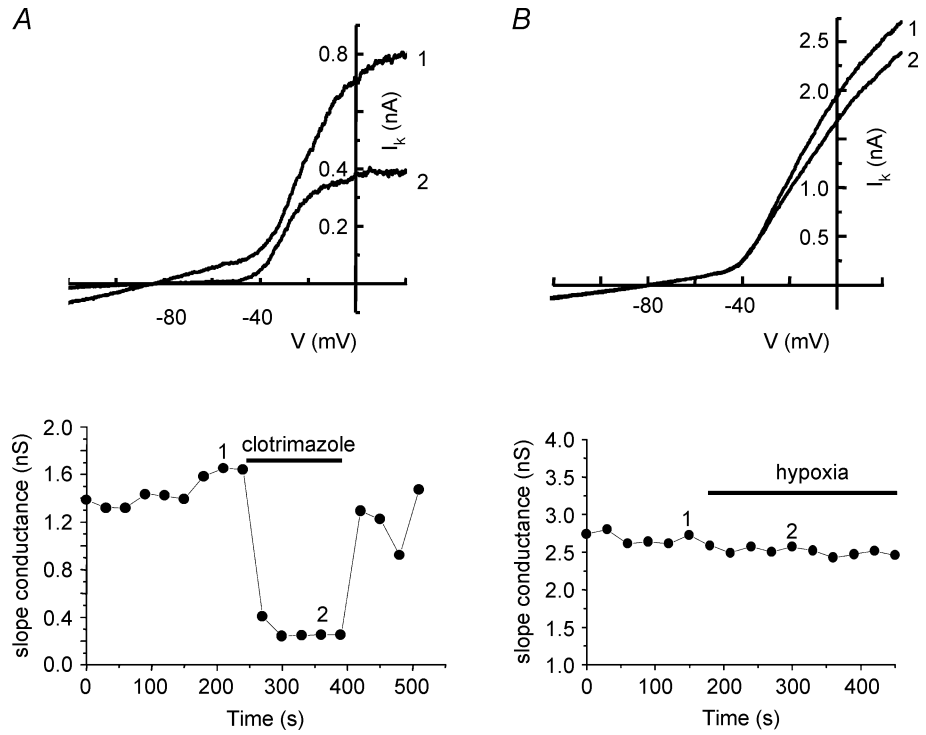


Figure 9. Hypoxia does not modulate IKCa1 channels
Clotrimazole (*A*), but not hypoxia (*B*), inhibits IKC1 currents in pre-activated human $CD3^+$ cells. K^+ currents were recorded with ramp pulse depolarization as described in Methods. The time courses of the effect of clotrimazole (145 nM) and hypoxia on the slope conductance are shown in the bottom panels. The continuous line indicates the time of exposure to the drug/hypoxia. The current traces corresponding to the time points indicated in the time-course graph are reported in the top panel. IKCa1 slope conductance was measured between −100 and −60 mV.

(Fanger *et al.* 2001) rather than the more physiologically relevant TCR engagement. More importantly, analysis of the data in other studies is often presented as the population average and not at the single-cell level, where significant variation can often be found (Hess *et al.* 1993; Guse, 1998).

The individual cell variability in O_2 sensitivity we observed upon anti-CD3/CD28 stimulation might also result from the compartmentalized distribution of the Kv1.3 channels in the T cell membrane. It has been recently shown that Kv1.3 channels co-localize with the TCR–CD3 antigen–receptor complex in the immunological synapses together with other components of the early T cell receptor signalling (Panyi *et al.* 2004*a*). This special organization might lead to a more efficient suppression of T cell activation upon Kv1.3 channel blockade than whole-cell channel ratios would indicate. Kv1.3 recruitment into the synapses might vary in different T cells, amplifying hypoxia sensitivity only when it occurs.

Such hypotheses require further testing. Nonetheless, our data suggest that the physiological consequences of Kv1.3 inhibition cannot be deduced from relative numbers of channels alone, especially when that inhibition is acute instead of chronic and when intermediate signalling molecules might be required to modulate the channel activity.

Overall, the data presented here indicate that hypoxia, by inhibiting Kv1.3 channels and depolarizing the membrane potential, blunts the TCR-activated increase in cytoplasmic Ca^{2+}. Similar responses are conserved as the hallmark of O_2-sensitive cells such as carotid body type I cells (the O_2-sensitive cells of the carotid body) and pulmonary artery smooth muscle cells (Lopez-Barneo *et al.* 2001). In these cells hypoxia triggers a characteristic sequence of events: inhibition of O_2-sensitive K^+ (Ko_2) currents, membrane depolarization and changes in cytoplasmic [Ca^{2+}] (Lopez-Barneo *et al.* 2001). Our results indicate that a similar sequence of events is found in the majority of human T cells although degrees of O_2 sensitivity appear to be present among different T cells.

References

Bortner CD, Gomez-Angelats M & Cidlowski JA (2001). Plasma membrane depolarization without repolarization is an early molecular event in anti-fas-induced apoptosis. *J Biol Chem* **276**, 4304–4314.

Brown JM (2000). Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies. *Mol Med Today* **6**, 157–162.

Cahalan MD, Wulff H & Chandy KG (2001). Molecular properties and physiological roles of ion channels in the immune system. *J Clin Immunol* **21**, 235–252.

Caldwell CC, Kojima H, Lukashev D, Armstrong J, Farber M, Apasov SG & Sitkovsky MV (2001). Differential effects of physiologically relevant hypoxic conditions on T lymphocyte development and effector functions. *J Immunol* **167**, 6140–6149.

Cayabyab FS, Khanna R, Jones OT & Schlichter LC (2000). Suppression of the rat microglia Kv1.3 current by src-family tyrosine kinases and oxygen/glucose deprivation. *Eur J Neurosci* **12**, 1949–1960.

Conforti L, Petrovic M, Mohammad D, Lee S, Ma Q, Barone S & Filipovich AH (2003). Hypoxia regulates expression and activity of kv1.3 channels in T lymphocytes: a possible role in T cell proliferation. *J Immunol* **170**, 695–702.

Fanger CM, Neben AL & Cahalan MD (2000). Differential Ca^{2+} influx, KCa channel activity, and Ca^{2+} clearance distinguish Th1 and Th2 lymphocytes. *J Immunol* **164**, 1153–1160.

Fanger CM, Rauer H, Neben AL, Miller MJ, Wulff H, Rosa JC, Ganellin CR, Chandy KG & Cahalan MD (2001). Calcium-activated potassium channels sustain calcium signaling in T lymphocytes. Selective blockers and manipulated channel expression levels. *J Biol Chem* **276**, 12249–12256.

Ghanshani S, Wulff H, Miller MJ, Rohm H, Neben A, Gutman GA, Cahalan MD & Chandy KG (2000). Up-regulation of the IKCa1 potassium channel during T-cell activation. Molecular mechanism and functional consequences. *J Biol Chem* **275**, 37137–37149.

Grynkiewicz G, Poenie M & Tsien RY (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**, 3440–3450.

Guse AH (1998). Ca^{2+} signaling in T-lymphocytes. *Crit Rev Immunol* **18**, 419–448.

Hess SD, Oortgiesen M & Cahalan MD (1993). Calcium oscillations in human T and natural killer cells depend upon membrane potential and calcium influx. *J Immunol* **150**, 2620–2633.

Hockel M & Vaupel P (2001). Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* **93**, 266–276.

Ishii TM, Silvia C, Hirschberg B, Bond CT, Adelman JP & Maylie J (1997). A human intermediate conductance calcium-activated potassium channel. *PNAS* **94**, 11651–11656.

Kalman K, Pennington MW, Lanigan MD, Nguyen A, Rauer H, Mahnir V, Paschetto K, Kem WR, Grissmer S, Gutman GA, Christian EP, Cahalan MD, Norton RS & Chandy KG (1998). ShK-Dap22, a potent Kv1.3-specific immunosuppressive polypeptide. *J Biol Chem* **273**, 32697–32707.

Kojima H, Sitkovsky MV & Cascalho M (2003). HIF-1 alpha deficiency perturbs T and B cell functions. *Curr Pharm Des* **9**, 1827–1832.

Koo GC, Blake JT, Talento A, Nguyen M, Lin S, Sirotina A *et al.* (1997). Blockade of the voltage-gated potassium channel Kv1.3 inhibits immune responses in vivo. *J Immunol* **158**, 5120–5128.

Launay P, Cheng H, Srivatsan S, Penner R, Fleig A & Kinet JP (2004). TRPM4 regulates calcium oscillations after T cell activation. *Science* **306**, 1374–1377.

Leonard RJ, Garcia ML, Slaughter RS & Reuben JP (1992). Selective blockers of voltage-gated K^+ channels depolarize human T lymphocytes: mechanism of the antiproliferative effect of charybdotoxin. *Proc Natl Acad Sci U S A* **89**, 10094–10098.

Lewis JS, Lee JA, Underwood JC, Harris AL & Lewis CE (1999). Macrophage responses to hypoxia: relevance to disease mechanisms. *J Leukoc Biol* **66**, 889–900.

Lewis RS (2001). Calcium signaling mechanisms in T lymphocytes. *Annu Rev Immunol* **19**, 497–521.

Lewis RS & Cahalan MD (1995). Potassium and calcium channels in lymphocytes. *Annu Rev Immunol* **13**, 623–653.

Loeffler DA, Juneau PL & Heppner GH (1991). Natural killer-cell activity under conditions reflective of tumor micro-environment. *Int J Cancer* **48**, 895–899.

Loeffler DA, Juneau PL & Masserant S (1992). Influence of tumour physico-chemical conditions on interleukin-2-stimulated lymphocyte proliferation. *Br J Cancer* **66**, 619–622.

Loeffler DA, Keng PC, Baggs RB & Lord EM (1990). Lymphocytic infiltration and cytotoxicity under hypoxic conditions in the EMT6 mouse mammary tumor. *Int J Cancer* **45**, 462–467.

Lopez-Barneo J, Pardal R & Ortega-Saenz P (2001). Cellular mechanism of oxygen sensing. *Annu Rev Physiol* **63**, 259–287.

Lukashev D, Caldwell C, Ohta A, Chen P & Sitkovsky M (2001). Differential regulation of two alternatively spliced isoforms of hypoxia-inducible factor-1 alpha in activated T lymphocytes. *J Biol Chem* **276**, 48754–48763.

Makino Y, Nakamura H, Ikeda E, Ohnuma K, Yamauchi K, Yabe Y, Poellinger L, Okada Y, Morimoto C & Tanaka H (2003). Hypoxia-inducible factor regulates survival of antigen receptor-driven T cells. *J Immunol* **171**, 6534–6540.

Marincola FM, Jaffee EM, Hicklin DJ & Ferrone S (2000). Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol* **74**, 181–273.

Naldini A & Carraro F (1999). Hypoxia modulates cyclin and cytokine expression and inhibits peripheral mononuclear cell proliferation. *J Cell Physiol* **181**, 448–454.

Palacios EH & Weiss A (2004). Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene* **23**, 7990–8000.

Panyi G, Vamosi G, Bacso Z, Bagdany M, Bodnar A, Varga Z, Gaspar R, Matyus L & Damjanovich S (2004*a*). Kv1.3 potassium channels are localized in the immunological synapse formed between cytotoxic and target cells. *Proc Natl Acad Sci U S A* **101**, 1285–1290.

Panyi G, Varga Z & Gaspar R (2004*b*). Ion channels and lymphocyte activation. *Immunol Lett* **92**, 55–66.

Prakriya M & Lewis RS (2001). Potentiation and inhibition of Ca^{2+} release-activated Ca^{2+} channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP_3 receptors. *J Physiol* **536**, 3–19.

Rader RK, Kahn LE, Anderson GD, Martin CL, Chinn KS & Gregory SA (1996). T cell activation is regulated by voltage-dependent and calcium-activated potassium channels. *J Immunol* **156**, 1425–1430.

Schindl R, Kahr H, Graz I, Groschner K & Romanin C (2002). Store depletion-activated CaT1 currents in rat basophilic leukemia mast cells are inhibited by 2-aminoethoxydiphenyl borate. Evidence for a regulatory component that controls activation of both CaT1 and CRAC (Ca^{2+} release-activated Ca^{2+} channel) channels. *J Biol Chem* **277**, 26950–26958.

Spisek R, Chevallier P, Morineau N, Milpied N, Avet-Loiseau H, Harousseau JL, Meflah K & Gregoire M (2002). Induction of leukemia-specific cytotoxic response by cross-presentation of late-apoptotic leukemic blasts by autologous dendritic cells of nonleukemic origin. *Cancer Res* **62**, 2861–2868.

Stokes L, Gordon J & Grafton G (2004). Non-voltage-gated L-type Ca^{2+} channels in human T cells: pharmacology and molecular characterization of the major alpha pore-forming and auxiliary beta-subunits. *J Biol Chem* **279**, 19566–19573.

Swain S (2003). Lymphocyte effector functions. Lymphocyte heterogeneity – is it limitless? *Curr Opin Immunol* **15**, 332–335.

Verheugen JA & Vijverberg HP (1995). Intracellular Ca^{2+} oscillations and membrane potential fluctuations in intact human T lymphocytes: role of K^+ channels in Ca^{2+} signaling. *Cell Calcium* **17**, 287–300.

Whiteside TL (1998). Immune cells in the tumor microenvironment. Mechanisms responsible for functional and signaling defects. *Adv Exp Med Biol* **451**, 167–171.

Wulff H, Beeton C & Chandy KG (2003*a*). Potassium channels as therapeutic targets for autoimmune disorders. *Curr Opin Drug Discov Devel* **6**, 640–647.

Wulff H, Calabresi PA, Allie R, Yun S, Pennington M, Beeton C & Chandy KG (2003*b*). The voltage-gated Kv1.3 K^+ channel in effector memory T cells as new target for MS. *J Clin Invest* **111**, 1703–1713.

Zhu WH, Conforti L, Czyzyk-Krzeska MF & Millhorn DE (1996). Membrane depolarization in PC-12 cells during hypoxia is regulated by an O_2-sensitive K^+ current. *Am J Physiol* **271**, C658–665.

Zuckerberg AL, Goldberg LI & Lederman HM (1994). Effects of hypoxia on interleukin-2 mRNA expression by T lymphocytes. *Crit Care Med* **22**, 197–203.

Acknowledgements

We thank Drs E. Gruenstein and J. N. Lorenz for technical help and advice during the Ca^{2+} imaging experiments. We also thank Dr A. Fomina for sharing her expertise in recording CRAC currents. This work was supported by NIH grant CA95286 to L.C.