Disruption of Kv1.3 Channel Forward Vesicular Trafficking by Hypoxia in Human T Lymphocytes

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Background: Chronic hypoxia decreases Kv1.3 channel surface expression in T lymphocytes.


Conclusion: Hypoxia disrupts AP1/clathrin mediated forward trafficking of Kv1.3 from the trans-Golgi to the plasma membrane.

Significance: Mechanism for reduced immune surveillance by T cells in hypoxic tumors.

Hypoxia in solid tumors contributes to decreased immunosurveillance via down-regulation of Kv1.3 channels in T lymphocytes and associated T cell function inhibition. However, the mechanisms responsible for Kv1.3 down-regulation are not understood. We hypothesized that chronic hypoxia reduces Kv1.3 surface expression via alterations in membrane trafficking. Chronic hypoxia decreased Kv1.3 surface expression and current density in Jurkat T cells. Inhibition of either protein synthesis or degradation and endocytosis did not prevent this effect. Instead, blockade of clathrin-coated vesicle formation and forward trafficking prevented the Kv1.3 surface expression decrease in hypoxia. Confocal microscopy revealed an increased retention of Kv1.3 in the trans-Golgi during hypoxia. Expression of adaptor protein-1 (AP1), responsible for clathrin-coated vesicle formation at the trans-Golgi, was selectively down-regulated by hypoxia. Furthermore, AP1 down-regulation increased Kv1.3 retention in the trans-Golgi and reduced Kv1.3 currents. Our results indicate that hypoxia disrupts AP1/clathrin-mediated forward trafficking of Kv1.3 from the trans-Golgi to the plasma membrane thus contributing to decreased Kv1.3 surface expression in T lymphocytes.

Hypoxia, a decrease in oxygen availability, can be encountered by cells both in physiological and pathological conditions. A hypoxic microenvironment originates in pathological sites such as wounds, solid tumors, atheromatous plaques, and joints with rheumatoid arthritis (1–3). The hypoxic microenvironment is responsible for altered gene expression and function of the affected cells (1). In solid tumors hypoxia has been associated with poor prognosis and resistance to conventional cancer treatments (3, 4).

Mobilization of T lymphocytes is required for mounting a competent immune response and circulating immune cells are subjected to varying degrees of oxygen tension during their maturation life cycle and during their sojourn in the tissues (1). It has been shown that hypoxia adversely affects T cell activation (1, 5). Exposure to hypoxia inhibits differentiation of naive T cells into cytotoxic T cells and decreases the production of cytokines such interleukin-2 (IL-2) and interferon-γ (IFNγ) (1, 3, 6). The reduced immune function associated with hypoxia is particularly troublesome in conditions such as solid tumors when an immune response would be beneficial to fight the disease in hand. Indeed, hypoxia and the tumor microenvironment contribute to the decrease in immune surveillance in solid tumors (3). Thus, it is critical to understand the mechanisms responsible for decreased immune function in hypoxia.

Cells respond to a hypoxic microenvironment through O2-sensitive pathways associated with hypoxia inducible factors, HIF-1α and HIF-2α, and ion channels (1, 4, 7, 8). In particular, ion channels have been shown to be the effector proteins that translate the detection of hypoxia into functional responses in many cell types including chemosensitive and immune cells.

T lymphocytes express voltage-dependent Kv1.3 channels that are involved in T cell activation and cytokine production (9). In fact, Kv1.3 channels control the membrane potential of resting and chronically activated human T cells. Inhibition of Kv1.3 channels causes the T cell membrane to depolarize, thus inhibiting Ca2+ signaling and associated downstream functions, such as cytokine release and proliferation, thereby ultimately suppressing T cell activation (9). Previous studies from our laboratory have shown that hypoxia inhibits Ca2+ signaling and proliferation in human T lymphocytes and that this effect is due to the down-regulation of Kv1.3 channels (5, 10, 11). Specifically, we showed that chronic hypoxia induced a decrease in Kv1.3 protein levels and functional channels in T cells, which was associated with a decrease in T cell proliferation (5). The down-regulation of Kv1.3 protein levels in hypoxia was not associated with changes in Kv1.3 mRNA expression suggesting that it occurred post-transcriptionally (5). Although regulation of Kv1.3 expression plays such an important role in hypoxia, the underlying regulatory mechanisms are unknown.
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Alterations in Kv1.3 biosynthesis and/or membrane trafficking may be involved in the channel down-regulation by chronic hypoxia. Although little is known about Kv1.3 protein trafficking, like other eukaryotic proteins, Kv1.3 channels are synthesized in the endoplasmic reticulum (ER)\(^2\) (12). In general, newly synthesized proteins are subsequently transported to the Golgi and finally to the cell surface along secretory pathways (forward trafficking) (13, 14). Proteins from the plasma membrane are internalized by endocytosis into endosomes from where they are either recycled back to the plasma membrane or are targeted to the lysosome for protein degradation (reverse trafficking) (13, 15). Potassium channels appear to follow similar trafficking pathways and, like other proteins, their surface expression depends on the balance between the forward and reverse trafficking pathways that depend on the protein itself, chaperone proteins, and signaling pathways (16–19). Thus, diverse trafficking mechanisms regulate surface expression of channels at the plasma membrane. The effect of hypoxia on ion channel trafficking is poorly understood.

The current study was undertaken to understand the mechanisms that mediate the decrease in Kv1.3 functional channels in human T lymphocytes during prolonged exposure to hypoxia. In the present study, we present evidence that chronic hypoxia reduces Kv1.3 protein surface expression in T cells via disruption of Kv1.3 vesicular trafficking from the trans-Golgi to the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Fetal bovine serum was obtained from Thermo Fisher Scientific (Rockford, IL). For cell culture, HEPES, RPMI, penicillin, and streptomycin were from Thermo Fisher Scientific. Odyssey Bio-Rad. Antibodies against the intracellular epitope of Kv1.3 (catalog number AB5178) were obtained from Millipore (Billerica MA). Antibodies against the extracellular epitope of the Kv1.3 channel (EC-Kv1.3) were obtained from either Millipore (catalog number AB5589) or Sigma (catalog number P4497). Antibodies against the \(\gamma\) subunit of adaptor protein (AP) 1 and \(\alpha\) subunit of AP2 were obtained from BD Biosciences, whereas monoclonal and polyclonal antibodies to \(\beta\)-actin were from Alpha Diagnostic International (San Antonio, TX). Sheep anti-human TGN-46 antibody, a trans-Golgi marker was procured from AbD Serotec (Oxford UK). Mouse anti-human antibodies against LAMP-2 and pan-cadherin were purchased from Abcam (Cambridge, MA). Alexa Fluor\textsuperscript{®} 680, 488, and 568 anti-rabbit and 555 anti-mouse secondary antibodies were obtained from Molecular Probes (Invitrogen), IRDye\textsuperscript{®} 800CW anti-mouse from Rockland, whereas DyLight\textsuperscript{®} anti-sheep 488 and 649 antibodies were from AbD Serotec (Oxford, UK).

**Cell Culture**—Jurkat T cells (clone E6-1) were obtained from American Tissue Culture Collection (ATCC, Manassas, VA). Cells were grown in suspension in RPMI supplemented with 10% FBS, 200 units/ml of penicillin, 200 \(\mu\)g/ml of streptomycin, and 10 \(\mu\)M HEPES. Cells were grown in a humidified incubator in the presence of 5% \(\text{CO}_2\) at 37 °C. Cells no later than passage 10 were used. Cell viability was determined by trypan blue exclusion.

**Exposure of Cells to Hypoxia**—To study the effect of chronic hypoxia, Jurkat cells were cultured in a water-jacketed \(\text{CO}_2\) incubator (Thermo Fisher Scientific) at 37 °C for 24 h as previously described (5). Briefly, cells were maintained in 1% \(\text{O}_2\), 5% \(\text{CO}_2\), and equilibrated with \(\text{N}_2\) for hypoxia, whereas normoxic cells were cultured in 21% \(\text{O}_2\) and 5% \(\text{CO}_2\).

**DNA Constructs**—A photostable human Kv1.3 channel N terminus construct fused to green fluorescence protein (EGFP-Kv1.3) was obtained as previously described (20). Galactosyltransferase (GalT) fused to cyan fluorescence protein (ECFP-GalT, plasmid number 11937) was obtained from Addgene Inc. (Cambridge MA) (21, 22).

**siRNAs**—Silencer\textsuperscript{®} Pre-designed siRNA against the \(\gamma\) subunit of AP1 adaptor protein (AP1\textsuperscript{G} gene, siRNA ID s1144, catalog number 4392421) and scrambled sequence siRNA (negative control 1, catalog number 4390843) were obtained from Ambion (Life Science Technologies, Carlsbad, CA). Predesigned siRNA against Kv1.3 channel was obtained from Santa Cruz Biotechnology Inc.

**Transfection**—10 × 10\(^6\) Jurkat cells were transfected using the Amaza Nucleofector Kit (Lonza, Cologne, Germany). Either 3 \(\mu\)g of plasmids or 100 \(\mathrm{nM}\) siRNAs were transfected using program T-14 as per the manufacturer’s instructions. In experiments where cells were cotransfected with siRNAs and GFP, pMAX GFP supplied in the Amaza Nucleofector Kit was cotransfected along with the siRNA at a GFP:siRNA ratio of 1:10. The efficiency of transfection was 50%.

**RT-qPCR**—Total RNA was isolated using the E.Z.N.A. total RNA Isolation System was purchased from Omega Bio-Tek (Norcross, GA) and TaqMan\textsuperscript{®} Reverse Transcription Reagents were obtained from Applied Biosystems/Invitrogen. Assay-on-Demand\textsuperscript{TM} Primers and 2X TaqMan Fast Universal PCR Master Mix for qPCR expression were obtained from Applied Biosystems/Invitrogen (Carlsbad CA). Pierce IP lysis buffer, HalTM protease inhibitor mixture, and bicinchoninic assay reagents were from Thermo Fisher Scientific. Odyssey\textsuperscript{®} blocking buffer was procured from Licor Biosciences (Lincoln, NE). Molecular weight markers for Western blot analysis were from Bio-Rad. Antibodies against the intracellular epitope of Kv1.3

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\(2\) The abbreviations used are: ER, endoplasmic reticulum; AP, adaptor protein; ARF, ADP-ribosylation factor; BafA1, bafilomycin A1; BFA, brefeldin A; CHX, cycloheximide; DYN, dynasore; EC-Kv1.3, extracellular Kv1.3; Lact, lactacystin; siAP1, silencing RNA against adaptor protein 1; Scr, scrambled sequence siRNA; GaT, galactosyltransferase; pQPCR, quantitative PCR; TGN, trans-Golgi network; ROI, region of interest; EGFP, enhanced green fluorescent protein; FRAP, fluorescence recovery after photobleaching; BF, brightfield; ECFP, enhanced cyan fluorescent protein.
(GAPDH) were obtained from Applied Biosystems. The RT-qPCR was set up in a 48-well plate by adding 40 ng of cDNA, 1× TaqMan Fast Universal PCR Master Mix (Applied Biosystems), and 1 μl of Assay-on-Demand primers. All samples were run in quadruplicates. GAPDH was used as an internal control. RT-qPCR was cycled in Applied Biosystems StepOne™ Real-time PCR system (Applied Biosystems). \(C_T\) values were measured using StepOne software version 2.1 (Applied Biosystems). \(C_T\) values for AP1 were normalized against measured \(C_T\) values for GAPDH and the \(\Delta\Delta C_T\) values were calculated. Relative quantity values, representing the fold-change in AP1 gene expression as compared with controls, were calculated as the \(2^{-\Delta\Delta C_T}\) values.

**Electrophysiology**—Patch clamp experiments were performed using Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in whole cell configuration as previously described (5). Kv1.3 currents were recorded with an external pipette solution composed of (mM): 134 KCl, 2 CaCl2, 2 MgCl2, 10 glucose, and 10 HEPES, pH 7.4. The pipette solution was composed of (mM): 134 KCl, 2 CaCl2, 2 MgCl2, 10 EGTA, and 10 HEPES, pH 7.4. The Kv1.3 currents were measured by depolarizing voltage steps to +50 mV from a holding potential of −80 mV every 30 s. The digitized signals were stored and analyzed using pClamp 9 software (Axon Instruments, Foster City, CA). Experiments were conducted at room temperature (22 °C). To calculate the inactivation time constant (\(\tau\)), the current decay was fitted to a single exponential equation, \(f(t) = A \exp(-t/\tau) + C\).

**On-Cell Western Assay**—Jurkat T cells were incubated in normoxia or hypoxia and plated on poly-L-lysine-coated coverslips in a 24-well tissue culture plate. The cells were then fixed in 3.7% formaldehyde for 20 min and blocked for 60 min at room temperature with a blocking buffer containing PBS and 10% FBS. The blocking buffer was removed and cells were incubated overnight with gentle rocking at 4 °C in EC-Kv1.3 antibody diluted with the blocking buffer. The following day, cells were permeabilized by incubation with blocking buffer containing 0.1% Triton X-100 for 30 min at room temperature. Cells were exposed to β-actin antibody for 60 min at room temperature and subsequently washed with 0.1% Tween 20 in PBS (PBS-T). Coverslips were incubated for 60 min at room temperature with the appropriate labeled secondary antibodies (Alexa Fluor 630 anti-rabbit and IRDye 800CW anti-mouse) diluted in blocking buffer. The coverslips were washed with PBS-T for 5 min with gentle shaking. The coverslips were imaged by scanning simultaneously at 700 and 800 nm with the LI-COR Odyssey® infrared scanner (Licor Biosciences) at 169-μm resolution.

**Image Analysis**—Images were acquired and analyzed using the Odyssey version 3.0 software (Licor Biosciences) as described earlier (11). EC-Kv1.3 was imaged at 700 nm, whereas actin was visualized at 800 nm. The measured fluorescence for EC-Kv1.3 in each coverslip was normalized to the measured fluorescence for β-actin in the same coverslip. The normalized Kv1.3 intensities were averaged and are represented as a fold-change relative to the normoxic controls for each experiment.

The efficacy of the On-Cell Western Assay in measuring Kv1.3 surface expression was confirmed in Jurkat cells transfected with siRNA against Kv1.3 (siKv1.3) (supplemental Fig. S1). Knockdown of Kv1.3 by siKv1.3 induced a 75% decrease in functional Kv1.3 channels (supplemental Fig. S1B). The On-Cell Western Assay measured a 44% reduction in Kv1.3 surface proteins (supplemental Fig. S1A). If we consider that in the On-Cell Western Assay we measure the surface protein levels in the whole population of cells, of which only 50% or less have incorporated the siRNAs, whereas in electrophysiological experiments we record only from transected cells visualized by GFP expression, comparable results were obtained with these techniques.

**Western Blot**—Jurkat cell lysates were prepared as described earlier (5). The protein content of the lysates was measured using the BCA Protein Assay. Cell lysates were mixed with 3× SDS sample buffer (New England Biolabs) and an equal amount of protein was loaded onto each lane of a 4–12% Tris glycine gel (Invitrogen) in a X Cell SureLock™ Gel System (Invitrogen). The protein bands were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) in a blotting system (Bio-Rad). The membrane was blocked with Odyssey Blocking Buffer (Licor Biosciences) at room temperature for 1 h and subsequently incubated overnight at 4 °C with primary antibodies against AP1 or AP2 along with β-actin. The blots were washed thoroughly with PBS-T and incubated with Alexa Fluor 680 anti-rabbit and IRDye 800CW anti-mouse secondary antibodies for 60 min at room temperature. The blots were then visualized with the LI-COR Odyssey infrared scanner at 169-μm resolution. Densitometric analysis for the protein expression was done by Odyssey version 3.0 software as described earlier (5).

**Immunofluorescence and Confocal Microscopy**—Jurkat cells, plated on poly-L-lysine-coated coverslips, were fixed in 3.7% formaldehyde, permeabilized with a solution of PBS and 10% FBS containing 0.1% Triton X-100 for 20 min at room temperature, and blocked with a solution of PBS containing 10% FBS for 1 h. Cells were probed overnight at 4 °C with the following antibodies diluted with the blocking solution: rabbit anti-human TGN-46, mouse anti-human LAMP-2. The following day, cells were incubated for 60 min with appropriate fluorescent secondary antibodies as indicated in the figure legends. Cells were washed thoroughly with PBS-T washing solution and coverslips were mounted on glass slides using Fluormount G (Fisher Scientific, Fairlawn, NJ). Cells were visualized by confocal microscopy (Leica Scanning Microscope LSM 510 Meta, Carl Zeiss MicroImaging GmbH) using a ×100 oil immersion objective lens at room temperature. Data were obtained using the “Multi Track” option of the microscope to exclude the cross-talk between the channels and analyzed by LSM Image browser (Carl Zeiss MicroImaging GmbH).

**Analysis of Confocal Images for Colocalization**—Colocalization was measured by correlation analysis using the LSM Image Browser. Briefly, the z-stack in the focal plane of the trans-Golgi (indicated by the TGN-46 antibody staining) was selected. A circle was drawn around the stained trans-Golgi, which was designated as the region of interest (ROI). For cells stained with LAMP-2 antibody, the entire cell was selected as the ROI. The image threshold and background were adjusted and thePear-
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son correlation coefficient ($R$) for the Kv1.3 and TGN-46/LAMP-2 channels was calculated using the image acquisition software. The values for $R$ were between −1 and +1 and were interpreted as described previously (23). $R$ of +1 indicated strong colocalization, whereas −1 indicated the absence of colocalization. For cells co-transfected with siRNAs and GFP, only cells demonstrating positive GFP staining were considered for analysis.

Fluorescence Recovery after Photobleaching (FRAP)—Jurkat cells were co-transfected with ECFP-GalT and EGFP-Kv1.3 DNA constructs in RPMI medium without phenol red. Twelve h after transfection, cells were incubated in either normoxia or hypoxia for 24 h. Cells were then seeded on gelatin-coated glass coverslips and returned to normoxia or hypoxia for 30 min before FRAP experiments. Live cell imaging was done by confocal microscopy (Laser Scanning Microscope LSM 510 Meta, Carl Zeiss Microlmaging GmbH) using a ×100 oil immersion objective lens at room temperature. GFP and CFP signals were collected in separate channels using specific band pass filters at wavelengths of 467–499 and 505–550 nm for CFP and GFP, respectively. CFP was excited at 458 nm, whereas GFP was excited at 488 nm. The confocal pinhole was set at ≤1 airy unit. Images were acquired sequentially to minimize the cross-talk between the two channels. The Golgi region was identified by ECFP-GalT staining. This region was selected as the ROI and FRAP studies were performed in this ROI for the EGFP-Kv1.3 channel. Pre-bleach images were taken for the GFP channel at 1% laser intensity. Subsequently, the ROI was bleached at 100% laser intensity using 100 iterations and the fluorescence recovery in the bleached ROI ($F_{ROI}$) was recorded by scanning at 1% laser intensity at 5-s intervals for 6–10 min. Simultaneously, the fluorescence intensity of an unbleached region showing similar GFP fluorescence as the $F_{ROI}$ and of similar size ($F_{CTR}$), and background fluorescence ($F_{BKG}$) were recorded.

Analysis of FRAP Studies—Fluorescence intensities of the time lapse images were acquired using the LSM Image Browser. $F_{BKG}$ levels were subtracted from the $F_{ROI}$ and $F_{CTR}$ values. For each time point, the $F_{ROI}$ was normalized against $F_{CTR}$. The normalized $F_{ROI}$ at the time of photobleach was set as the zero time point ($F_0$). The normalized fluorescence recovery ($F_{REC}$) at each time point ($t$) was calculated from the formula $F_{REC} = (F_{zero} - F_0)/(1 - F_0)$. For each experiment, the normalized fluorescence recovery was plotted as a function of time and fitted to an exponential equation, $F(t) = A \times (1 - e^{-t/\tau})$, where $A$ represents the Mobile fraction and $\tau$ represents the time constant of fluorescence recovery. The half-life of fluorescence recovery ($t_{1/2}$), was calculated by $t_{1/2} = \tau \ln 2$. $p < 0.05$ was considered as statistically significant.

RESULTS

Prolonged Exposure to Hypoxia Decreases Kv1.3 Surface Expression—We have previously shown that chronic hypoxia decreases Kv1.3 total protein levels and Kv1.3 current density in human T cells (5). A decrease in functional channels could be due to a decrease in surface Kv1.3 protein levels and/or a decrease in active channels in the membrane. We thus conducted experiments to determine whether exposure to prolonged hypoxia (chronic hypoxia) alters the surface expression of Kv1.3 channels in Jurkat T cells. Exposure to chronic hypoxia was achieved by maintaining the cells in an incubator with 1% $O_2$ (5% $CO_2$, balanced with $N_2$) at 8 mm Hg) at 37 °C (5). Throughout the manuscript the term “hypoxia” refers to chronic hypoxia achieved as described above. Hypoxia did not alter the cell viability (all viability data are presented in supplemental Table S1). Hypoxia had instead a significant effect on Kv1.3 surface expression (Fig. 1). Surface Kv1.3 protein levels in Jurkat cells were measured by On-Cell Western Assay (11, 24). As described under “Experimental Procedures,” intact (nonpermeabilized) cells were probed with a specific Kv1.3 antibody against amino acid residues 211–224 on the extracellular loop of the Kv1.3 protein, thus providing us with a true measure of Kv1.3 protein expression on the cell surface. The specificity of this antibody was previously established (25). As shown in Fig. 1A, hypoxia reduced Kv1.3 protein surface expression, whereas the levels of β-actin remained unchanged and was thus used as internal control. On average there was a 40 ± 5% (n = 4, $p < 0.001$) reduction in Kv1.3 surface expression in hypoxia (Fig. 1B). Whole cell voltage-clamp experiments were performed to determine whether the decrease in Kv1.3 protein surface expression in hypoxia was associated with a decrease in functional Kv1.3 channels. As shown in Fig. 1C, hypoxia induced a significant ~48% decrease in current

![FIGURE 1. Hypoxia inhibits Kv1.3 channel surface expression and current amplitude in T cells. A and B, effect of hypoxia on Kv1.3 surface protein expression. Kv1.3 surface expression was measured in Jurkat cells by On-Cell Western Assay after exposure to normoxia (N) and hypoxia (H) for 24 h. A, representative images of cells stained with rabbit extracellular anti-Kv1.3 (EC-Kv1.3) antibody (red) and mouse anti-β-actin antibody (green). The merged images are shown in the bottom panels. Shown here is an experiment representative of four independent experiments each in quadruplicate. B, average Kv1.3 surface expression in normoxia and hypoxia. The relative intensity of Kv1.3 for each coverslip was calculated as a ratio of the measured integrated intensities of Kv1.3 and β-actin for the same coverslip and normalized to the value of mean relative intensity calculated in normoxia. The data represent mean ± S.E. from 4 independent experiments, each in quadruplicate. C, effect of hypoxia on Kv1.3 currents. Left, representative Kv1.3 currents recorded in Jurkat cells maintained in normoxia and hypoxia for 24 h. Kv1.3 currents were elicited by depolarizing voltage steps from −80 mV (holding potential, HP) to +50 mV every 30 s. Right, average Kv1.3 current density under normoxic (n = 9) and hypoxic conditions (n = 9).]
density (current density = current/capacitance) \( n = 9, p = 0.01 \). These currents were identified as Kv1.3 currents by their characteristic biophysical properties (voltage-dependent activation and C-type inactivation) and sensitivity to specific blockers (5). There was no change in cell capacitance between normoxic and hypoxic cells. The capacitances for cells maintained in normoxia and hypoxia were \( 3.8 \pm 0.4 \) \( n = 9 \) and \( 4.6 \pm 0.4 \) \( p \) \( n = 9, p = 0.17 \), respectively. Furthermore, there was no change in the time constants of inactivation (\( \tau \)) \( 268 \pm 26 \) ms \( n = 9 \) in normoxia and \( 261 \pm 15 \) ms in hypoxia \( n = 8, p = 0.82 \). These findings are consistent with previous reports from our laboratory that showed a 47% decrease in total Kv1.3 protein levels and a 42% decrease in current density in Jurkat as well as primary human T cells by chronic hypoxia (5).

Next, we performed further experiments to assess whether the effect of hypoxia was specific to Kv1.3 proteins and it was not a generalized effect on plasma membrane proteins. The effect of hypoxia on Kv1.3 surface expression was compared with that on the plasma membrane protein cadherin (26, 27). We found no effect of hypoxia on cadherin. As shown in supplemental Fig. S2, we observed a reduction in Kv1.3 surface protein levels in hypoxia, whereas the levels of cadherin remained unchanged. Overall, there was a 29 \( \pm 1 \% \) \( n = 3, p < 0.001 \) reduction in Kv1.3 surface expression in hypoxia when the Kv1.3 surface protein levels were normalized to cadherin. This is comparable with the effect observed when \( \beta \)-actin was used as the internal control \( p = 0.32 \) thus confirming the validity of \( \beta \)-actin as a housekeeping protein in our experiments. Overall, these data indicate that down-regulation of functional Kv1.3 channels during chronic hypoxia results from a decrease in Kv1.3 protein surface expression.

Hypoxia Does Not Alter Kv1.3 Protein Synthesis—A decrease in surface protein expression can be due to alterations in protein synthesis/degradation and/or trafficking. We first examined whether the decrease in Kv1.3 surface expression in hypoxia was due to the inhibition of protein synthesis. Cycloheximide (CHX) inhibits protein biosynthesis in the ER by blocking the elongation phase during protein translation (28). Jurkat cells were maintained in hypoxia and normoxia in the presence of CHX (500 \( \mu \)g/ml) or vehicle (controls) for 24 h and the expression of surface Kv1.3 and \( \beta \)-actin were determined (Fig. 2A). Treatment with CHX reduced \( \beta \)-actin (31 \( \pm 3 \% \) inhibition, \( n = 3, p < 0.001 \)) and Kv1.3 (41 \( \pm 4 \% \) inhibition, \( n = 3, p < 0.001 \)) levels in normoxia, indicative of effective protein synthesis inhibition (Fig. 2, A and B). Exposure to hypoxia induced a significant inhibition of Kv1.3 surface expression in control cells (35 \( \pm 6 \% \), \( n = 3, p < 0.001 \)) as well as CHX-treated cells (39 \( \pm 6 \% \), \( n = 3, p = 0.002 \)) (Fig. 2C). Exposure to CHX did not alter cell viability either in normoxia or hypoxia (supplemental Table S1). Overall, there was no significant difference in the percentage inhibition of Kv1.3 surface expression by hypoxia in control and CHX-treated cells \( p = 0.9 \), Fig. 2D). These data indicate that the reduction of Kv1.3 surface expression in hypoxia is not due to inhibition of protein synthesis.

Disruption of Forward Trafficking Mediates the Reduction in Kv1.3 Surface Protein Levels during Hypoxia—Experiments were conducted to study whether the effect of hypoxia on the expression of Kv1.3 channels is due to alterations in forward

**FIGURE 2. Reduced Kv1.3 protein surface expression during hypoxia is not due to decreased protein synthesis.** A–D, effect of protein synthesis inhibition on Kv1.3 surface levels. A, representative experiment of cells preincubated with 500 \( \mu \)g/ml of CHX (+ CHX) or vehicle (−CHX) for 30 min and then exposed for 24 h to either normoxia (N) or hypoxia (H). CHX was present throughout the experiment. Kv1.3 surface expression and \( \beta \)-actin levels were measured by On-Cell Western Assay with EC-Kv1.3 (red) and anti-\( \beta \)-actin (green) antibodies. Merged images are shown in the bottom panels. Each panel depicts a single coverslip from each condition in a representative experiment. Three independent experiments were performed, each in quadruplicate. B, average \( \beta \)-actin and surface Kv1.3 down-regulation by CHX. Fluorescence intensities were measured for Kv1.3 and \( \beta \)-actin in control (−CHX) and CHX-treated cells in normoxia. The data are reported as normalized to control values. C and D, effect of CHX on Kv1.3 surface expression inhibition in hypoxia. C, the relative intensity of EC-Kv1.3 for each coverslip was calculated as a ratio of the measured integrated intensities of EC-Kv1.3 and \( \beta \)-actin (see "Experimental Procedures"). The calculated relative intensities for EC-Kv1.3 are normalized to control (−CHX). D, the percentage inhibition of Kv1.3 surface expression in hypoxia in the presence (+ CHX) or absence (−CHX) of CHX was calculated from normalized values in panel C. For panels B–D, the data represent mean \( \pm \) S.E. for 3 independent experiments, each in quadruplicate.

The role of proteasomal degradation in the down-regulation of Kv1.3 surface expression during hypoxia was studied by preincubating Jurkat cells with the proteasomal inhibitor Lact (5 \( \mu \)M) (31). As shown in Fig. 3, B and D, the percentages of inhibition of Kv1.3 surface expression in hypoxia were not significantly different in vehicle-treated control and BafA1-treated cells (Fig. 3, A and D). The percentages of inhibition were 22 \( \pm 2 \% \) in controls \( n = 3 \) and 27 \( \pm 2 \% \) \( n = 3 \) \( p = 0.24 \) in BafA1-treated cells. Exposure to BafA1 did not alter cell viability (supplemental Table S1). Overall, these data ruled out a role of endocytosis in the reduction of Kv1.3 surface expression in hypoxia.

The role of proteasomal degradation in the down-regulation of Kv1.3 surface expression during hypoxia was studied by preincubating Jurkat cells with the proteasomal inhibitor Lact (5 \( \mu \)M) (31). As shown in Fig. 3, B and D, the percentages of inhibition of Kv1.3 surface expression in hypoxia were not significantly different in vehicle-treated control (23 \( \pm 1 \% \), \( n = 3 \)) and Lact-treated cells (30 \( \pm 3 \% \), \( n = 3 \), \( p = 0.12 \)). Treatment with Lact did not reduce the viability of Jurkat cells (supplemental Table S1). These data exclude proteasomal degradation in the reduction of Kv1.3 surface expression during hypoxia.

The involvement of forward trafficking pathways in down-regulating Kv1.3 surface expression during hypoxia was studied with BFA. BFA is a widely used inhibitor of the trans-Golgi (32).
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FIGURE 3. Disruption of forward trafficking, and not endocytosis, mediates the reduction in Kv1.3 surface protein levels in hypoxia. A–D, Jurkat cells were exposed to 1 μM bafilomycin A1 (+/−BafA1), 5 μM lactacystin (+/−Lact), or 10 μM brefeldin A (+BFA) for 30 min and then incubated in normoxia (N) or hypoxia (H) for 24 h in presence of the inhibitors. Control cells (−BafA1, −Lact, and −BFA) were treated with vehicle. Relative Kv1.3 surface protein expression was determined by On-Cell Western Assay. The relative intensity of EC-Kv1.3 for each coverslip was calculated as a ratio of the measured integrated intensities of EC-Kv1.3 and β-actin for (A) BafA1, (B) Lact, and (C) BFA. The calculated relative intensities for EC-Kv1.3 are normalized to control (−/−BafA1, −/−Lact, and −/−BFA). D, the percentage inhibition of Kv1.3 surface expression in hypoxia in the presence or absence of the inhibitors (+/−BafA1, +/−Lact, and +/−BFA) is reported. The data represent mean ± S.E. of n (number of independent experiments) each in quadruplicate (n = 3 for BafA1, n = 3 for Lact, and n = 4 for BFA).

Jurkat cells were pre-treated with BFA (10 μM) or vehicle (control) and incubated in normoxia or hypoxia. Treatment with BFA did not decrease the viability of Jurkat cells (supplemental Table S1). As shown in Fig. 3, C and D, treatment with BFA abrogated the inhibition of Kv1.3 surface expression during hypoxia that was otherwise observed in vehicle-treated control cells. The percentage inhibition of Kv1.3 surface expression in hypoxia was 22 ± 0% in vehicle-treated cells and −3 ± 5% in BFA-treated cells (n = 4, p = 0.004). These data indicate that exposure to hypoxia disrupts forward trafficking of Kv1.3 protein from the Golgi thereby down-regulating Kv1.3 surface expression.

Confocal microscopy experiments confirmed the involvement of the trans-Golgi in the reduction of Kv1.3 surface expression during hypoxia. Jurkat cells were incubated in normoxia and hypoxia and stained with antibodies against Kv1.3 and TGN-46, a trans-Golgi marker (33). Hypoxia increased colocalization of Kv1.3 and TGN-46 (Fig. 4). The colocalization was quantified by calculating the Pearson correlation coefficient (23). The correlation coefficient in hypoxia, 0.38 ± 0.06 (n = 42), was significantly higher than the correlation coefficient determined in normoxia, −0.02 ± 0.07 (n = 40; p < 0.001), thereby indicating a higher degree of colocalization between TGN-46 and Kv1.3 in hypoxia.

The involvement of the Golgi was further substantiated by FRAP experiments. To perform FRAP studies we needed to express a fluorescence-tagged Kv1.3 and a marker of the Golgi to visualize the Golgi in these very small cells. We thus co-expressed EGFP-Kv1.3 and ECFP-GalT, a resident enzyme of the Golgi complex (21, 34). The ability of Kv1.3 channels to accumulate in the Golgi during hypoxia was maintained in cells that overexpressed EGFP-Kv1.3 and ECFP-GalT (supplemental Fig. S3). The correlation coefficient of EGFP-Kv1.3 and ECFP-GalT was significantly higher in hypoxia (0.47 ± 0.06, n = 5) than in normoxia (0.01 ± 0.05, n = 5, p < 0.001).

FRAP experiments were thus performed in EGFP-Kv1.3 and ECFP-GalT transfected Jurkat cells (Fig. 5). During confocal microscopy, using separate channels with specific band pass filters to image the Jurkat cells enabled us to identify the Golgi region tagged by ECFP-GalT and EGFP-Kv1.3 (Fig. 5A). The EGFP-Kv1.3 in the region of the Golgi was marked as ROI (Fig. 5A), photobleached, and the fluorescence recovery was measured by time-lapse confocal microscopy until a plateau was reached. As shown in Fig. 5B, in both normoxia and hypoxia, following photobleaching, cells regained ~90% of their fluorescence. However, cells incubated in hypoxia exhibited a slower fluorescence recovery as compared with normoxic cells (Fig. 5B). Analysis of the FRAP data showed equal EGFP-Kv1.3 Mobile fractions in normoxia and hypoxia, but a significantly higher t1/2 in hypoxia as compared with normoxia (Table 1). These data indicate that the trafficking of Kv1.3 proteins through the Golgi is altered during hypoxia.

The exclusion of a lysosomal involvement in down-regulation of Kv1.3 surface expression in hypoxia was further confirmed by confocal microscopy. Jurkat cells were incubated in normoxia or hypoxia in the presence of the protease inhibitor leupeptin (5 μg/ml) to inhibit lysosomal degradation and stained with antibodies against Kv1.3 and LAMP-2, a specific marker of lysosomal membranes (29, 35). No colocalization was observed between Kv1.3 and LAMP-2 in either normoxia or hypoxia-treated cells (Fig. 6). There was no significant difference in the correlation coefficients in hypoxia (−0.03 ± 0.04, n = 52) and normoxia (0.02 ± 0.03, n = 44; p = 0.3) as shown in Fig. 6B, thereby indicating lack of colocalization between LAMP-2 and Kv1.3 in both normoxia and hypoxia.

Overall, these data indicate that forward trafficking of Kv1.3 proteins is altered during hypoxia and a greater number of Kv1.3 channels is retained in the trans-Golgi during hypoxia, thereby resulting in a decrease in their surface expression. Experiments were thus conducted to determine the mechanisms mediating Kv1.3 retention in the trans-Golgi.

The Clathrin-coated Vesicle Formation Process Is Involved in Down-regulation of Kv1.3 Surface Expression during Hypoxia—Formation of clathrin-coated vesicles occurs during protein secretion from the trans-Golgi toward the plasma membrane as well as during endocytosis of plasma membrane proteins. During budding of the clathrin-coated vesicles at the trans-Golgi as well as at the plasma membrane, a large GTPase dynamin is involved in scission of the neck of the invaginated clathrin pits (13). To determine whether a defect in clathrin-dependent vesicle formation is involved in the alteration of Kv1.3 channel surface expression in hypoxia, Jurkat cells were treated with the specific dynamin inhibitor DYN (80 μM) and the degree of Kv1.3 surface expression inhibition in hypoxia was compared with that of vehicle-treated control cells (36). As shown in Fig. 7, A and B, the hypoxic inhibition of Kv1.3 surface expression
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FIGURE 4. Hypoxia induces Kv1.3 channel retention in the trans-Golgi. A, colocalization of Kv1.3 and TGN-46 determined by immunofluorescence. Jurkat cells were exposed to normoxia (N) and hypoxia (H) for 24 h, fixed in 3.7% formaldehyde, permeabilized, and labeled with rabbit anti-Kv1.3 and sheep anti-TGN46, followed by Alexa Fluor 568-conjugated goat anti-rabbit (red) and DyLight 488-conjugated donkey anti-sheep (green) secondary antibodies. Images were obtained by confocal microscopy, as described under “Experimental Procedures.” Scale bars indicate 10 μm. Cells marked by arrows in the merged images are magnified in the extreme right panels to highlight the differences in the colocalization of TGN-46 and Kv1.3 in normoxia and hypoxia. Increased colocalization (yellow) between TGN-46 (green) and Kv1.3 (red) was observed in hypoxia. B, colocalization of Kv1.3 and TGN-46 fluorescence signals was quantified by correlation analysis (see “Experimental Procedures”). Data represent mean ± S.E. for correlation coefficients calculated from individual cells in normoxia (n = 40) and hypoxia (n = 42).

(20 ± 1%, n = 3) observed in control cells was abrogated by dyn (∼0.9 ± 3%, n = 3; p = 0.004).

These data indicate that a clathrin- and dynamin-dependent mechanism mediates the reduction of Kv1.3 surface expression in hypoxia. This finding combined with the observation that forward trafficking and not reverse trafficking is involved in Kv1.3 deregulation in hypoxia indicates that hypoxia disrupts the clathrin-mediated vesicle formation necessary to transport Kv1.3 channels from the trans-Golgi to the membrane.

Hypoxia Inhibits Expression of AP1 Adaptor Protein—The process of clathrin-coated vesicle formation in the trans-Golgi differs from that at the plasma membrane by the presence of two distinct AP (13, 15, 37, 38). AP1 is recruited at the trans-Golgi and initiates formation of clathrin-coated vesicles at this site, whereas AP2 participates in the formation of clathrin-coated vesicles at the plasma membrane (13). APs are protein complexes formed by four subunits (13, 37). AP1 is formed by γ, β1, σ1, and μ1 subunits, whereas AP2 is formed by α, β2, σ2, and μ2 subunits (37, 39). We observed that hypoxia selectively down-regulates AP1γ, but not AP2α expression (Fig. 8).

Hypoxia induced a 23 ± 1% reduction in AP1γ gene expression (n = 3, p < 0.001) (Fig. 8A) and a 24 ± 2% (n = 3; p = < 0.001) reduction in AP1γ protein levels (Fig. 7B). However, hypoxia had no significant effect on AP2α protein levels (Fig. 8B). These results suggest that reduced AP1 expression in hypoxia may be responsible for inhibiting Kv1.3 protein secretion.

Down-regulation of AP1 Mimics the Effect of Hypoxia on Kv1.3 Surface Expression—To determine whether AP1 is involved in Kv1.3 down-regulation in hypoxia, the effect of AP1 knockdown on the expression of Kv1.3 channels was investigated. A siRNA against the AP1γ subunit (siAP1) was used to knockdown the AP1γ subunit gene (AP1G1). The efficacy and selectivity of siAP1 is shown in supplemental Fig. S4. Transfection with siAP1 produced a reduction in mRNA levels of 50 ± 1% (n = 3) after 48 h and 40 ± 3% (n = 3) reduction in protein levels after 72 h. However, siAP1 did not reduce AP2 protein levels. If we take into account that the efficiency of transfection by nucleoporation of Jurkat T cells is in the range of 40–60% as reported previously, we anticipate a significant down-regulation of AP1 protein in the transfected cells (40). We thus proceeded to compare the degree of Kv1.3 localization in the trans-Golgi in siAP1 and scrambled sequence siRNA (Scr)-transfected cells both in normoxia and hypoxia (Fig. 9). Jurkat cells were transfected with siAP1 or Scr, exposed to either normoxia or hypoxia for 24 h and stained with Kv1.3 and TGN-46 antibodies. Because the cells were co-transfected with siRNAs and a GFP plasmid at a 10:1 ratio, only the transfected cells, recognized by the GFP fluorescence, were analyzed. As shown in Fig. 9, A and B, down-regulation of AP1 in normoxia produced a significant increase in Kv1.3 and TGN-46 colocalization indicative of increased Kv1.3 retention in the trans-Golgi. The average correlation coefficient for Scr in normoxia was 0.16 ± 0.04 (n = 14), whereas the correlation was significantly increased to 0.43 ± 0.04 in siAP1-transfected cells (n = 10; p < 0.0001). The retention of Kv1.3 in the trans-Golgi in AP1 knockdown experiments resulted in a decrease in the expression of functional Kv1.3 channels at the plasma membrane (Fig. 9C). The average current density in Scr-transfected cells was
parable with Kv1.3 channel retention in the trans-Golgi shown in nontransfected cells (Fig. 4). The Kv1.3-TGN-46 correlation coefficient in Sgr cells in hypoxia was 0.47 ± 0.05 (n = 10), which is significantly higher than that measured in normoxia in Sgr-transfected cells (p < 0.001). A similar degree of Kv1.3 localization in the trans-Golgi was observed in siAP1 cells in hypoxia with a correlation coefficient of 0.44 ± 0.07 (n = 6; p = 0.74 compared with Scr in hypoxia) (Fig. 9B). No additive effect in the Kv1.3 channel retention in the trans-Golgi was observed in AP1-silenced cells incubated in hypoxia as compared with hypoxic Sgr controls. The findings presented in Figs. 8 and 9 indicate that reduced AP1 expression is involved in the decrease of functional Kv1.3 channels during hypoxia.

**DISCUSSION**

Ion channels are key mediators of the response to hypoxia in many cell types including T lymphocytes (5, 8). Whereas the effect of acute hypoxia on ion channel activity has been well investigated, limited information exists on the effect of chronic hypoxia on ion channel expression and function (5, 8, 41–43). In human T lymphocytes the expression of functional Kv1.3 channels is decreased after prolonged hypoxia and the channel down-regulation is associated with a decrease in cell proliferation (5). The studies presented herein define the mechanism that mediates Kv1.3 channel down-regulation in chronic hypoxia. We report that hypoxia inhibits Kv1.3 forward trafficking from the trans-Golgi to the plasma membrane via down-regulation of clathrin-coated vesicle formation. This indicates that Kv1.3 retention in the trans-Golgi is a mechanism for down-regulating T cell function in hypoxia. To our knowledge, this is the first time that alterations in forward vesicular trafficking have been implicated in the modulation of ion channel expression during hypoxia.

In the present study we reported that the decrease in functional Kv1.3 channels in Jurkat cells after prolonged exposure to hypoxia is due to inhibition of Kv1.3 protein cell surface expression. This is consistent with previous studies from our laboratory that have shown that both Kv1.3 total protein levels and functional channels were inhibited by chronic hypoxia in primary T lymphocytes and Jurkat cells (5). This effect was specific to Kv1.3 channels (the protein levels of SK2, a K<sub>Ca</sub> channel expressed in Jurkat T cells, were unchanged) and to the Kv1.3 pore forming α subunit (the expression of the auxiliary Kvβ2 subunit was unchanged) (5). Herein we showed that the decrease in Kv1.3 surface expression during hypoxia is also specific of this membrane protein because, in accordance with previous studies, the expression of the plasma membrane protein cadherin remained unaltered (26, 27).

Like other surface proteins, the expression level of ion channels in the membrane is determined by the delicate balance between biosynthesis, membrane delivery, and endocytic recycling and degradation (14, 44). Various evidences exist that ion channel regulation can occur during biogenesis. It has been reported that reduction of functional Kv1.3 channels by the accessory subunit KCNE4 (Kv channel subfamily E member 4) is due to retention of Kv1.3 channels in the ER (45). Furthermore, chronic hypoxia reduced the expression of hERG membrane proteins by blocking the protein maturation in the ER

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<th>Table 1: Effect of hypoxia on Kv1.3 mobile fraction and half-life</th>
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<td>Effects of FRAP studies showing Mobile fractions and half-life of fluorescence recovery calculated as described under “Experimental Procedures.” Data represent mean ± S.E. of four separate experiments both in normoxia and hypoxia.</td>
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<td>Mobile fraction</td>
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39.8 ± 6.0 (n = 8) and 23.2 ± 3.3 pA/pF (n = 9; p = 0.03) in siAP1-transfected cells. There was no change in cell capacitance: the measured capacitances were 3.8 ± 0.3 pF (n = 8) in Sgr-transfected cells and 3.7 ± 0.3 pF (n = 9) in siAP1-transfected cells (p = 0.76). Furthermore, there was no change in the time constants of inactivation (τ): 276.4 ± 18.8 ms (n = 8) in Sgr and 268.9 ± 22.6 ms in siAP1-transfected cells (n = 9; p = 0.81). These data indicate a decrease in the number of functional Kv1.3 channels in the plasma membrane. Thus, similar to hypoxia, silencing the AP1 gene produced retention of Kv1.3 in the trans-Golgi, which resulted in a decrease in the number of functional Kv1.3 channels in the plasma membrane.

Interestingly, knockdown of AP1 did not potentiate Kv1.3 retention in the trans-Golgi during hypoxia. Exposure to hypoxia results in the retention of Kv1.3 channels in the trans-Golgi in Sgr-transfected cells (Fig. 9, A and B) and this is com-

**FIGURE 5.** Hypoxia modulates Kv1.3 mobility in the Golgi. A, expression of ECFP-GalT and EGFP-Kv1.3 constructs in Jurkat cells. ECFP-GalT (blue) and EGFP-Kv1.3 (green) were visualized by confocal microscopy as described under “Experimental Procedures.” Shown here is a representative cell expressing both ECFP-GalT (GalT) and EGFP-Kv1.3 (Kv1.3). The Golgi region was identified by the ECFP-GalT fluorescence in the CFP channel and selected as ROI as shown by the circle in the extreme right panel. EGFP-Kv1.3 channels in the ROI were photobleached during the FRAP experiments. Scale bar indicates 5 μm. B, FRAP analysis of Kv1.3 channel mobility in the Golgi of Jurkat cells maintained either in normoxia (N) or hypoxia (H). Jurkat cells were transfected with ECFP-GalT and EGFP-Kv1.3 and incubated in normoxia and hypoxia for 24 h. FRAP image acquisition and analysis was performed as outlined under “Experimental Procedures.” Average normalized Golgi ECFP-Kv1.3 fluorescence recoveries in normoxia (▲, n = 4) and hypoxia (■, n = 4) are plotted as a function of time(s). Solid lines represent the curve fitting of normoxia and hypoxia data.
Contrary to these findings, we showed that blockade of Kv1.3 protein synthesis in the ER by CHX did not prevent the Kv1.3 surface expression inhibition induced by hypoxia. Similar to our findings, it was recently reported that modulation of KCNQ1 (Kv7.1) channel by the Kv channel subunit KCNE1 was not prevented by CHX, but required forward vesicular trafficking (47). In this study, we report evidence of the involvement of forward vesicular trafficking on Kv1.3 regulation during hypoxia. No involvement of reverse trafficking was observed. BafA1, which inhibits reverse trafficking by blocking endosomal vesicle maturation and lysosomal degradation, and the proteasomal inhibitor Lact, did not prevent the effect of hypoxia on Kv1.3. Furthermore, hypoxia did not induce accumulation of the Kv1.3 protein in the lysosomes. Although the mechanism of membrane trafficking of Kv1.3 channels is not known, there is evidence that several other channels like Kv1.5, Kv1.2, and hERG are internalized by endocytosis from the plasma membrane in a clathrin- and dynamin-dependent manner (16, 17, 19, 48). In addition to endocytosis of proteins from the plasma membrane, the clathrin- and dynamin-mediated vesicular transport is also responsible for the forward trafficking of membrane proteins from the trans-Golgi network (13, 15, 49). Although we observed that inhibition of dynamin (a GTPase responsible for the fission of the necks of clathrin-coated buds and formation of clathrin-coated vesicles) abrogated the effect of hypoxia on Kv1.3, thereby suggesting an involvement of the clathrin-mediated transport process, we also observed that inhibition of endosomal trafficking by BafA1 had no effect on Kv1.3 down-regulation in hypoxia (50). Instead, BFA abrogates the effect of hypoxia on Kv1.3 surface expression. BFA functions by inhibiting the binding of small G proteins of ADP-ribosylation factors (ARF1-GTP) and formation of vesicles at the trans-Golgi (32, 51). The BFA effect is exclusive to the trans-Golgi as BFA does not affect the number of clathrin-coated vesicles at the plasma membrane (52). Treatment with BFA ultimately decreases the expression of cell surface proteins, secretory proteins, lipids and viral proteins (53). In T cells, BFA was shown to block CD69 surface expression, but not intracellular CD69 expression indicating that BFA inhibits secretion of proteins to the cell surface, but has no effect on protein synthesis (54). Treatment with BFA blocked the forward trafficking of hERG channels in HEK293 cells and reduced hERG currents (48).

FRAP studies showed that trafficking of the Kv1.3 protein through the Golgi was altered during hypoxia. Although Kv1.3 channels maintained the ability to diffuse freely and rapidly through the Golgi as indicated by the high Mobile fractions both in normoxia and hypoxia, they displayed slower kinetics (21, 22, 55, 56). The slow fluorescence recovery during hypoxia could be due to impairment of either Kv1.3 trafficking from the ER to the Golgi and vice versa, or Kv1.3 trafficking from the Golgi to the plasma membrane. Our findings exclude the former possibility and indicate that clathrin-mediated forward trafficking is impaired in hypoxia. Although the trafficking of proteins between the ER to the Golgi is mediated by coat pro-
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**FIGURE 7.** Inhibition of clathrin-coated vesicle formation abrogates down-regulation of Kv1.3 surface expression in hypoxia. Jurkat cells were preincubated for 30 min with 80 μM dynasore (+DYN) or vehicle (−DYN) and exposed to either normoxia or hypoxia for 24 h. A, the relative intensity of EC-Kv1.3 for each coverslip was calculated as a ratio of the measured integrated intensities of EC-Kv1.3 and β-actin. The calculated relative intensities for EC-Kv1.3 are normalized to vehicle (−DYN). B, percent inhibition of Kv1.3 surface expression in hypoxia was calculated as described in the legend to Fig. 2. The data represent mean ± S.E. for n = 3, each experiment in quadruplicate.

**FIGURE 8.** Hypoxia inhibits the expression of AP1 adaptor protein. A, AP1 gene expression in Jurkat cells challenged with hypoxia (H) for 24 h. Cells incubated in normoxia (N) served as controls. AP1 γ gene expression was quantified by RT-qPCR (see “Experimental Procedures”). Shown here is the fold-change in AP1 γ gene expression relative to GAPDH expression. The data are normalized to normoxic controls. The data correspond to mean ± S.E. of 3 experiments, each in quadruplicate. B, Western blot analysis of AP1 γ subunit and AP2α subunit protein expression in Jurkat cells cultured in either normoxia (N) and hypoxia (H). 40 μg of protein lysate was loaded in each lane and the transferred proteins were probed with mouse anti-AP1/anti-AP2 antibodies, whereas rabbit anti-β-actin antibody was used as a loading control. C, quantification of AP1 and AP2 protein expression, shown in B, was performed by normalizing the integrated intensities of AP1/AP2 proteins with β-actin. The fold-change in protein expression is normalized to normoxia controls. Data represent mean ± S.E. from 3 independent experiments.

protein complexes I and II vesicles, a clathrin-mediated process is characteristic of forward trafficking from the trans-Golgi (13, 50, 57). We observed that Kv1.3 is retained in the trans-Golgi during hypoxia and that inhibition of molecular elements selectively involved in clathrin-coated vesicle formation such as dynamin abrogated the effect of hypoxia on Kv1.3 surface expression. These studies also revealed the molecular elements involved in hypoxic down-regulation of Kv1.3 surface expression.

A complex machinery is involved in vesicle formation and key elements are the clathrin-associated APs, which allow binding of clathrin to biological membranes (15, 37, 58). Two different mechanisms as well as two different APs are involved in the trans-Golgi and plasma membrane (37, 58, 59). AP1 is mainly present at the trans-Golgi, whereas AP2 is present at the plasma membrane (15, 37, 38, 59). AP1 is recruited at the trans-Golgi by ARF1-GTP, which binds the cargo to be secreted to the membrane and initiates formation of clathrin-coated vesicles (13, 59). On the other hand, the binding of AP2 to the plasma membrane is not regulated by ARFs, but is due to phosphatidylinositol 4,5-bisphosphate (59, 60). We report that hypoxia selectively inhibits the gene and protein expression of AP1, but not AP2. This raises the question whether the AP1 γ promoter has hypoxia regulatory elements. No evidence of this has yet been reported. We also showed that down-regulation of AP1 in normoxic cells mimics the effect of hypoxia on Kv1.3 expression and results in retention of Kv1.3 channels in the trans-Golgi and lowers expression of functional Kv1.3 channels in the plasma membrane. Furthermore, retention of Kv1.3 in the trans-Golgi by AP1 down-regulation was not potentiated in hypoxia indicating that AP1/clathrin-mediated vesicle formation regulates Kv1.3 expression during hypoxia. This finding also indicates that clathrin-coated vesicles contribute to the transport of newly synthesized Kv1.3 channels to the plasma membrane. To our knowledge, this is the first report of AP1 involvement in ion channel forward trafficking. This finding raises the question whether Kv1.3 has a recognition site for AP1/clathrin. Close evaluation of the Kv1.3 protein sequence reveals an ELGLLI sequence commencing at amino acid position 397, which is a sorting signal of the dileucine (D/E)XXL(L/I) motif (where X is any amino acid) that binds to the γ/α1 subunit of AP1 and is speculated to bind to the β subunit of AP2 as well (15, 38). Kv1.3 also possesses a YMYI amino acid sequence at position 529, which is the YXXØ (where Ø represents a bulky hydrophobic amino acid) sorting signal that binds to the μ subunit of AP1 and AP2 (15). At this stage we still do not know whether Kv1.3 channels are transported by AP1/clathrin vesicles directly to the plasma membrane or to early endosomes and from there to the plasma membrane via a recycling mechanism (61–64). Importantly, our results indicate that inhibition of the AP1-mediated secretory pathway participates in inhibition of the Kv1.3 surface expression in chronic hypoxia. Modulation of ion...
channels via secretory vesicular pathways by hypoxia, to our knowledge, has never been reported although regulation of vesicular forward trafficking by Rab11 G proteins has been implicated in modulation of integrin expression during hypoxia (65).

In conclusion, our data indicate that hypoxia disrupts AP1/clathrin-mediated forward trafficking of Kv1.3 from the trans-Golgi to the plasma membrane thus contributing to decreased Kv1.3 surface expression in T lymphocytes. The decrease in surface expression of these channels contributes to the decrease in T cell function and reduced immune surveillance in solid tumors (5). The studies we presented provide insights into the Kv1.3 protein trafficking mechanisms in hypoxia and reveal new pathways that could represent new targets in the development of novel therapies aimed to restore T cell function and immune surveillance in solid tumors.

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Membrane Trafficking of Kv1.3 in Hypoxia