Hypoxia-induced Acidosis Uncouples the STIM-Orai Calcium Signaling Complex

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Background: STIM proteins are calcium sensors controlling Orai calcium entry channels.

Results: Hypoxia causes decreased intracellular pH and prevents Orai channel activation in response to calcium store depletion.

Conclusion: Hypoxia and intracellular acidification prevent coupling of STIM to Orai channels.

Significance: pH-mediated uncoupling of STIM-Orai may protect cells from hypoxia-mediated calcium overload.

The endoplasmic reticulum Ca\(^{2+}\)-sensing STIM proteins mediate Ca\(^{2+}\) entry signals by coupling to activate plasma membrane Orai channels. We reveal that STIM-Orai coupling is rapidly blocked by hypoxia and the ensuing decrease in cytosolic pH. In smooth muscle cells or HEK293 cells coexpressing STIM1 and Orai1, acute hypoxic conditions rapidly blocked store-operated Ca\(^{2+}\) entry and the Orai1-mediated Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (I\(_{\text{CRAC}}\)). Hypoxia-induced blockade of Ca\(^{2+}\) entry and I\(_{\text{CRAC}}\) was reversed by NH\(_4\)\(^{+}\)-induced cytosolic alkalization. Hypoxia and acidification both blocked I\(_{\text{CRAC}}\) induced by the short STIM1 Orai-activating region. Although hypoxia induced STIM1 translocation into junctions, it did not disassociate the STIM1-Orai1 complex. However, both hypoxia and cytosolic acidosis rapidly decreased Förster resonance energy transfer (FRET) between STIM1-YFP and Orai1-CFP. Thus, although hypoxia promotes STIM1 junctional accumulation, the ensuing acidification functionally uncouples the STIM1-Orai1 complex providing an important mechanism protecting cells from Ca\(^{2+}\) overload under hypoxic stress conditions.

Store-operated Ca\(^{2+}\) channels are ubiquitously expressed in excitable and nonexcitable cells and function through the coordinated operation of two distinct proteins, STIM and Orai (1–3). STIM proteins are ER\(^{+}\) membrane proteins functioning as dynamic sensors of Ca\(^{2+}\) within the ER lumen (1, 4). Decreased luminal Ca\(^{2+}\) activates STIM proteins causing them to aggregate and translocate into junctions of ER closely juxtaposed with the plasma membrane (1, 2). Within these junctions, the activated STIM proteins directly couple with the highly Ca\(^{2+}\)-selective family of Orai channels, tethering and opening the channels to generate Ca\(^{2+}\) entry signals (1–3). The entering Ca\(^{2+}\) provides spatially restricted and longer term Ca\(^{2+}\) signals crucial in controlling cellular responses, including gene expression and cellular growth (5–7). STIM proteins also exert control over other target channels, including transient receptor potential (canonical) channels (8) and L-type Ca\(^{2+}\) channels (9, 10). Recent studies reveal that ER Ca\(^{2+}\) depletion is not the only stress condition that modulates STIM activation. STIM1 is activated in response to oxidant stress, reactive oxygen species inducing STIM1 translocation, and coupling to open Orai channels independent of ER Ca\(^{2+}\) store depletion (11, 12). STIM1 is also activated by small increases in temperature that cause STIM1 translocation into junctions without store depletion (13); however, the coupling of STIM1 to open Orai channels is blocked at the higher temperature, although subsequent cooling promotes functional coupling to open Orai channels (12–14).

The activation of STIM proteins by store depletion, oxidants, and temperature reveals their function as sensors of multiple stress conditions (12, 14). Oxygen depletion is another major cellular stress with profound pathophysiological significance in cancer, inflammation, and vascular diseases (15, 16). Acute responses to hypoxic stress in vascular, airway, cardiac, and neural tissues are crucial to effect rapid adaptive responses in response to oxygen deprivation (16, 17). Longer term responses to hypoxic stress involve changes in gene expression through transcriptional regulators such as the hypoxia-inducible factors that control key compensatory responses to protect tissues from sustained oxygen depletion (15). Whereas the short and long term responses to hypoxic stress are well known, the initial mechanisms for sensing hypoxia have remained poorly defined. Ion channels, in particular both K\(^{+}\) and Ca\(^{2+}\) channels, are considered key early mediators of hypoxic stress sensing (17);
however, whether they are direct sensors of decreased oxygen or are functionally indirectly modified remains unclear (17).

We reveal here that hypoxic stress rapidly and reversibly inactivates STIM/Orai-mediated Ca\(^{2+}\) entry and the associated highly selective Ca\(^{2+}\) release-activated Ca\(^{2+}\) current \((I_{\text{CRAC}})\). The effect is observed on opening of endogenous Orai channels functioning in primary smooth muscle cells and also on functional coupling between STIM1 and Orai1 proteins overexpressed in HEK293 cells. We reveal that the action of hypoxic stress on STIM-Orai coupling is mediated by a rapid hypoxia-induced decline in cytosolic pH. This hypoxia-induced acidification blocks the functional coupling between the STIM-Orai activating regions (SOAR) of STIM1 required for Orai1 channel gating. The results suggest that the electrostatic interaction known to mediate coupling between STIM1 and Orai1 may operate as a pH-sensing mechanism to control Ca\(^{2+}\) entry signals in response to hypoxia.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary cultures of hASMC were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Humans as described previously (18). Cells were grown in high glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified 5% CO\(_2\), 95% O\(_2\) incubator at 37 °C. Cells were passaged weekly and typically used through passages 7–8. Cell growth was arrested by replacing DMEM with low serum 0.8% medium 48 h before experiments. HEK293 cells were maintained in high glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified 5% CO\(_2\), 95% O\(_2\) incubator at 37 °C, as described earlier (19). Transfection of DNA into cells was achieved by electroporation using the Bio-Rad Xcel electroporation system as described previously (20).

**Induction of Hypoxia**—To induce hypoxia, the following three methods were used. (a) Control solutions (either for imaging or electrophysiology) were mixed with freshly prepared sodium dithionite \((Na_2S_2O_4)\) solution to give a final 1 mM concentration sufficient to reduce \(PO_2\) to low levels (21, 22); pH was adjusted to 7.4, and the solution was bubbled with 100% N\(_2\). (b) Normal media were bubbled for >30 min with 100% N\(_2\). (c) Media were bubbled for >30 min with 3% O\(_2\). The \(PO_2\) in media containing 1 mM dithionite was ~0 mM Hg, measured using an MT200A MitoCell Clark-type oxygen electrode (Strathkelvin Instruments).

**Cytosolic Ca\(^{2+}\) Measurements**—Ratiometric imaging of intracellular Ca\(^{2+}\) using cells loaded with fura-2 was measured as described previously (23, 24). All HEK293 or tracheal smooth muscle cells for these experiments were grown in round coverslips (25 mm) under normal tissue culture conditions, except where specified. Coverslips with cells were placed in a cation-safe solution composed of the following (in mM): 107 NaCl, 7.2 KCl, 1.2 MgCl\(_2\), 11.5 glucose, 20 HEPES-NaOH, pH 7.2, and loaded with fura-2/AM (2 \(\mu\)M final concentration) for 30 min at room temperature. Cells were washed, and de-esterification was allowed for a minimum of 30 min. Ca\(^{2+}\) measurements were made using a Leica DMI 6000B fluorescence microscope controlled by the Slidebook software (Intelligent Imaging Innovations, Denver, CO). Fluorescence emission at 505 nm was monitored while alternating excitation wavelengths between 340 and 380 nm at a frequency of 0.5 Hz; intracellular Ca\(^{2+}\) measurements are shown as 340:380 nm ratio obtained from groups (15–25) of single cells. External solutions were as follows (in mM): 135 NaCl, 5.4 KCl, 10 HEPES, 0.02 NaH\(_2\)PO\(_4\), 2 Mg\(^{2+}\), 10 glucose, pH 7.4. For ASMC, verapamil (10 \(\mu\)M) was added to the extracellular buffer to block the L-type channel. Measurements shown are representative of a minimum of three independent experiments.

**Electrophysiological Measurements**—Electrophysiological recordings were generated using conventional whole-cell patch clamp, as described previously (24), using cells grown on glass coverslips within the recording chamber. Immediately after establishing the whole-cell configuration, linear voltage ramps of 50-ms duration spanning the voltage range of −100 to +100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz. We used automatic capacitive and series resistance compensation of the EPC-10 amplifier (HEKA Electronics). PatchMaster and Origin software were used for acquisition and analysis. The temporal development of inward current (at −100 mV) was measured from the individual ramps. The pipette solution contained the following (in mM): 145 CsGlu, 10 HEPES, 10 BAPTA, 8 NaCl, 10 Mg\(^{2+}\), 2 Mg\(^{2+}\)-ATP, pH 7.2. The excess Mg\(^{2+}\) and ATP were used to ensure inhibition of endogenous TRPM7 channels. At the time of break-in, the extracellular solution contained 145 NaCl, 10 CsCl, 2.8 KCl, 10 HEPES, 10 glucose, pH 7.4. For smooth muscle patch clamp studies, verapamil (10 \(\mu\)M) and niflumic acid (30 \(\mu\)M) were included in the external solution to inhibit any residual L-type voltage Ca\(^{2+}\) channel activity and Cl\(^{−}\) currents, respectively. Osmolarity of all solutions was adjusted to 300 mosm/liter. 10 mM CaCl\(_2\) was added to the external solution after break-in. The maximal Ca\(^{2+}\) release-activated Ca\(^{2+}\) \((I_{\text{CRAC}})\) currents at −100 mV after leak subtraction were used for statistical analysis.

**Fluorescence Imaging and FRET Measurements**—Experiments were performed in HEK293 cells stably expressing Orai1-CPF and transiently transfected with STIM1–YFP, STIM2–YFP, YFP-SOAR, or STIM1ct–YFP. Fluorescence was examined with a Leica DMI 6000B fluorescence microscope equipped with CFP (438\(_{\text{Ex}}/483\(_{\text{Em}}\)), YFP (500\(_{\text{Ex}}/542\(_{\text{Em}}\)) and FRET\(_{\text{raw}}\) (438\(_{\text{Ex}}/542\(_{\text{Em}}\)) filters controlled by Slidebook 4.2 software (Intelligent Imaging Innovations). Images \(I_{\text{FRET}}\), \(I_{\text{YFP}}\) and \(I_{\text{CFP}}\) were obtained every 10–20 s at room temperature with a ×40 oil objective (N.A.1.35; Leica). Three-channel corrected FRET was calculated based on the following formula: \(F_{\text{corrected}} = F_{\text{raw}} - F_{\text{m}}/D_{\text{m}}F_{\text{CFP}} - F_{\text{m}}/D_{\text{m}}F_{\text{YFP}}\), where \(F_{\text{m}}\) represents the corrected total amount of energy transfer; \(F_{\text{m}}/D_{\text{m}}\) represents measured bleed through of YFP through the CFP filter (0.473); \(F_{\text{m}}/D_{\text{m}}\) represents measured bleed through of YFP through the CFP filter (0.049). To reduce variations caused by differences in expression levels of CFP, the above \(F_{\text{m}}\) values were normalized against donor fluorescence \((F_{\text{YFP}})\). To minimize the effect of variations of YFP expression levels on \(F_{\text{CFP}}\)-normalized FRET signals \((F_{\text{N}})\) and to show the relative changes as compared with resting levels, figures are shown as \(\Delta F_{\text{N}}/F_{\text{rest}}\). Fluorescence images and FRET analyses
shown are typical of at least three separate experiments. In control experiments, we used N-terminally (as opposed to C-terminally) labeled STIM1 coexpressed with Orai1-CFP and detected no measurable FRET signal upon store depletion or with dithionite or NH4+ addition, indicating that the FRET signals represent authentic STIM-Orai interactions. 1 mM dithionite caused only marginal changes in YFP and CFP fluorescence; hence, observed changes in FRET are not due to direct effects of pH, in agreement with studies showing minor changes in CFP-YFP FRET due to pH changes from 7.0 to 6.5 (25), as induced by 1 mM dithionite. Image analysis of puncta was undertaken using ImageJ and Slidebook 4.2.0.13 (Olympus). Background was subtracted from the raw image, and noise (less than five consecutive pixels) was filtered out. Images were imported into Slidebook, and the size and number of puncta were measured using the mask function. The density of puncta was obtained by dividing the number of puncta by the size of the cell. Relative cell size was measured from the cell profile using ImageJ. To reconstruct three-dimensional images of cells, CFP or YFP image stacks were collected at 1-µm intervals. The constrained iterative deconvolution function of the Slidebook software was used to minimize fluorescence contamination from out-of-focus planes. Cell images at the ER-PM junction were used for display.

**Measurement of Intracellular pH**—For intracellular pH measurement, confluent HEK293 or smooth muscle cells were detached with 0.04% trypsin, 0.02% EDTA. Cells were washed and incubated with the fluorescent pH indicator 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) at 4 °C for 20 min at 37 °C in HEPES buffer, pH 7.4, supplemented with 1% BSA. Prior to measurements, cells were rinsed and resuspended in BSA-free HEPES buffer and transferred into a glass cuvette. Fluorescence measurements were detected with the RatioMaster cuvette fluorescence system (Photon Technology International Inc.) with the following wavelengths: dual excitation fixed at 492 and 438 nm; emission wavelength at 525 nm. Calibration of BCECF fluorescence was performed in HEPES buffer using carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) and titrated with solutions of NaOH and HCl as described (26). Sodium propionate and ammonium chloride were added in the same buffers used for cell perfusion and were made the same day of the experiments.

**Mouse Aortic Ring Contraction**—3-Month-old male C57BL/6J mice were euthanized by cervical dislocation, and thoracic aortas were removed and placed in ice-cold PBS with calcium and magnesium. The aorta was then cut into four 2.5-mm rings using a sharp surgical blade. 40-µm diameter stainless steel wires were threaded through the rings and then mounted on the support brackets within the tissue organ bath of a Radnoti Wire Myograph System (Radnoti LLC) containing 5 ml of Krebs-Henseleit (KH) buffer in (mm): 118 NaCl, 4.75 KCl, 1.19 KH2PO4, 1.19 MgSO4, 2.54 CaCl2, 12.5 NaHCO3, 10.0 glucose, at 37 °C and oxygenated with 95%O2 and 5%CO2. The rings were stretched to a tension of 0.5 g to simulate normal transmural pressure. Rings were maintained at this tension as they equilibrated for 90 min. To test ring viability, 100 mM KCl was added to the baths. After contractions reached a plateau, rings were washed with KH until reaching a 0.5-g base-line tension. To empty stores, rings were challenged with phenylephrine (1 µM). At the contraction plateau, KH in baths was replaced with 5 ml of a modified Ca2+-free Krebs buffer and containing thapsigargin (TG) (2 µM) and verapamil (10 µM) to inhibit store refilling and L-type channels. Respectively. Upon achieving base-line tension, the Ca2+-free buffer was replaced with 5 ml of KH containing Ca2+. At the peak of contraction, KH in baths was replaced with 5 ml of KH containing verapamil, sodium dithionite (1 mM) gassed with 95% N2, 5% CO2. The same solution was gassed with 95% O2, 5% CO2 to reverse hypoxia.

**ATP Measurements**—A7r5 cells were grown in 12-well plates and treated with dithionite for 5 min (n = 4). Samples were prepared using the CellTiter-Glo® luminescent cell viability assay kit (Promega) according to the manufacturer’s instructions. ATP levels (luminescence) were measured in a 96-farad nontreated white microwell plate (Nunc) using VICTOR X5 2030 Multilabel reader (PerkinElmer Life Sciences).

**RESULTS**

Acute responses to hypoxia are observed in airway and systemic vascular smooth muscle cells, with hypoxia rapidly inducing relaxation of both cell types, which is physiologically important in promoting oxygen movement into tissues (27). Evidence indicates that hypoxia-induced relaxation is mediated by decreased Ca2+ entry, and in some vascular smooth muscle cells diminished O2 inhibits the function of L-type Ca2+ channels (17, 28). However, the mechanism by which hypoxia modifies Ca2+ entry is unknown (17). Considering the powerful role of STIM proteins in controlling multiple Ca2+ entry channels (9), we examined the actions of hypoxia on STIM-mediated Ca2+ signaling. Cultured hASMC perfused in Ca2+-free medium were treated with the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) pump blocker TG to deplete ER Ca2+ stores and activate STIM proteins (20). Re-addition of extracellular Ca2+ revealed substantial store-operated Ca2+ entry (SOCE), which was remarkably sensitive to acute hypoxic stress (Fig. 1A). Hypoxia was induced either by perfusion of low O2-containing media or using the O2 scavenger sodium dithionite (29). In cells perfused with hypoxic medium (30 min, 3% O2) for 2 min prior to Ca2+ addition, peak SOCE was reduced by ~50% (49.6 ± 5.27%, n = 32, p < 0.05) (Fig. 1, A and B). With cells in which hypoxia was induced with 1 mM dithionite added 2 min before Ca2+ re-addition, the SOCE peak amplitude was reduced by almost 90% (87.2 ± 4.15%, n = 43, p < 0.05) compared with normoxic conditions (Fig. 1, A and B). Although Orai channels are known to be opened by activated STIM proteins, other channels are also modified by store-dependent STIM activation (1, 30). The presence of verapamil (10 µM) on the superfusate during these experiments ensured that the L-type channels did not contribute to the Ca2+ entry. To assess the specific function of Orai channels, hASMC were transfected with the powerful dominant negative Orai1 E106A-CFP mutant in which a crucial Ca2+ pore-forming acidic residue is replaced (31, 32). This mutant almost completely inhibited SOCE (93.06 ± 3.15%, n = 6) (Fig. 1C). hASMC express all three Orai channel subtypes (33), and Orai1–E106A-CFP blocks each Orai subtype (34) but has no effect on L-type or TRPC channels.
channels (9). The action of Orai1-E106A-CFP indicates that in hASMC virtually all Ca\textsuperscript{2+} entries induced by store emptying are mediated by Orai channels.

Although the results suggested hypoxia altered Orai-mediated Ca\textsuperscript{2+} entry, the fura-2 measurements could not exclude effects on other Ca\textsuperscript{2+} handling processes. We therefore assessed whether hypoxia altered the inwardly rectifying Ca\textsuperscript{2+} entry current, \(I_{\text{CRAC}}\), specifically mediated by Orai channels (35). We measured \(I_{\text{CRAC}}\) in voltage-clamped single hASMC during passive depletion of Ca\textsuperscript{2+} stores induced by intracellular BAPTA (9). \(I_{\text{CRAC}}\) developed and became maximal (peak of 1.66 \pm 0.29 pA/picofarads, \(n = 5\)) within 3 min of break-in. The current was dependent on extracellular Ca\textsuperscript{2+} stores, and the I/V relationship revealed the typical inwardly rectifying nature of the highly Ca\textsuperscript{2+}-selective CRAC channel (Fig. 1D) with reversal potential of approximately +40 mV (35). Although small, the current was similar in magnitude to that observed in vascular smooth muscle cells (9). Addition of 1 mM sodium dithionite reduced the current by 58.8 \pm 9.1% (\(n = 5\), \(p < 0.05\)) without altering channel selectivity (Fig. 1, D and E). Dithionite is a powerful O\textsubscript{2} scavenger substantially reducing molecular O\textsubscript{2} (29); as a reducing agent, it can also release active oxygen species (29). To assess whether O\textsubscript{2} reduction was responsible for the effect, dithionite solutions were gassed with 16% O\textsubscript{2} for 30 min to restore O\textsubscript{2} content (29). The O\textsubscript{2} restoration reduced the inhibitory effect of dithionite on SOCE to \(-80\%) that observed with a control solution identically gassed with 16% O\textsubscript{2} (supplemental Fig. S1, A and B) indicating that the action of dithionite was through reduced O\textsubscript{2} levels. To determine whether SOCE and its reversal by hypoxia relate to changes in smooth muscle contraction, we measured tension development in mouse aortic rings (Fig. 1, F and G). Application of phenylephrine caused a large contraction primarily because of Ca\textsuperscript{2+} release. Store depletion was maintained by application of TG, and addition of verapamil ensured there were no functioning L-type channels. Readdition of Ca\textsuperscript{2+} resulted in contraction mediated by SOCE (36), and subsequent addition of 1 mM dithionite caused rapid relaxation. Bubbling O\textsubscript{2} into the medium in the presence of dithionite resulted in recovery of contraction as O\textsubscript{2} was restored.

To more directly examine hypoxia on store-operated Ca\textsuperscript{2+} channels, we used HEK293 cells overexpressing both STIM1 and Orai1 (20, 23, 32). In these cells, following Ca\textsuperscript{2+} store depletion with 2 \(\mu\)M TG, Ca\textsuperscript{2+} readdition resulted in very high levels of Ca\textsuperscript{2+} entry (32). As in hASMC, perfusion with hypoxic medium (gassed with 3% O\textsubscript{2}) for 2 min caused a substantial decrease in Ca\textsuperscript{2+} entry (51.0 \pm 11.16\% \(n = 45\)) (Fig. 1I and supplemental Fig. S2A). Addition of 1 mM dithionite at the same
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FIGURE 2. Inhibition of $I_{\text{CRAC}}$ by hypoxia is mediated by cellular acidification. A, time course of $I_{\text{CRAC}}$ development in STIM1/Orai1-transfected HEK293 cells. 50 $\mu$M 2-APB was added at arrow. B, $I_{\text{CRAC}}$, in STIM1/Orai1-HEK293 with brief additions of 1 mM dithionite (Na-Dit; bars) and Ca$^{2+}$-free external medium (bar). Inset, I/V relationships at the times indicated by arrowheads. C and D, Intracellular pH measurements in BCECF-loaded human ASMC (C) or STIM1/Orai1-HEK293 cells (D); dithionite (1 or 5 mM) or ammonium chloride (20 mM) additions were made as shown. E, $I_{\text{CRAC}}$ in STIM1/Orai1-HEK293 with additions of 1 mM dithionite and 20 mM NH$_4^+$ (bars). Inset, I/V relationships at the times indicated by arrowheads. F, changes in intracellular pH in BCECF-loaded human STIM1/Orai1-HEK293 cells induced by 50, 100, and 150 mM propionate and 20 mM NH$_4^+$ additions (arrows). G, $I_{\text{CRAC}}$ in STIM1/Orai1-HEK293 with additions of 50 mM propionate and 20 mM NH$_4^+$ (bars). Inset, I/V relationships at the times indicated by arrowheads.

time as Ca$^{2+}$ readdition also caused a substantial reduction in SOCE (69.25 ± 7.66%, n = 63) (Fig. 1I and supplemental Fig. S2B), and cells treated for 2 min with dithionite before Ca$^{2+}$ readdition showed almost complete inhibition of SOCE activity (Fig. 1, H and J) (89.5 ± 6.65% n = 52) compared with normoxic controls.

The rapid actions of dithionite on SOCE suggested that the effect of hypoxia is either to directly block Orai channels or to reverse the coupling between STIM and Orai required to maintain Orai channel activation (1). The high CRAC current density (10–20 pA/picofarads) in the STIM1/Orai-expressing HEK293 cells allowed for more accurate examination of the actions of hypoxia. $I_{\text{CRAC}}$, induced by BAPTA became maximal within ~100–200 s (Fig. 2A), the time scale for store depletion and accumulation of STIM1 within ER-PM junctions (1, 24, 35). 2-Aminodiphenyl borate (2-APB, 50 $\mu$M) caused a biphasic response, including brief activation followed by rapid inhibition (Fig. 2A), typical of its action on CRAC channels (37, 38). Application of 1 mM dithionite rapidly inhibited $I_{\text{CRAC}}$, and this inhibition was reversed upon dithionite removal (Fig. 2B). This reversible inhibition could be repeated, and subsequent removal of external Ca$^{2+}$ substantially reduced $I_{\text{CRAC}}$, reflecting the Ca$^{2+}$ selectivity of the current (Fig. 2A). I/V curves for the trace (Fig. 2B) revealed channel properties were not altered by dithionite. Application of hypoxic medium gassed with N$_2$ also inhibited $I_{\text{CRAC}}$ (supplemental Fig. S3). The effects of this passive O$_2$ reduction were slower than with the active O$_2$ scavenger, dithionite, and the I/V curves again revealed no alteration in the channel properties.

A crucial question was how this profound effect of hypoxic stress is mechanistically sensed by the STIM-Orai signaling pathway. Hypoxia is known to cause rapid acidification of many cell types, including smooth muscle (27, 39) and cardiac (40) and neural cells (41) as they change from oxidative to glycolytic metabolism. Longer term hypoxia in actively growing cancer cells results in a powerful means to adapt to acidification (16, 42). The rapid actions of hypoxia on pH led us to investigate whether changes in intracellular pH were involved in the action of hypoxia on STIM/Orai-mediated Ca$^{2+}$ entry. Initially, we examined the effects of hypoxia on pH in cells loaded with the ratiometric fluorescence pH indicator. In hASMC, application of 1 mM dithionite resulted in a rapid decrease in intracellular pH (pH$_i$) from a resting level of approximately pH 7.0 to a new level of ~6.6 (Fig. 2C). Application of 20 mM ammonium chloride (NH$_4^+$) caused rapid cytoplasmic alkalinization because of proton sequestration. In STIM1/Orai-transfected HEK293 cells, application of 1 mM dithionite also caused a rapid initial pH change followed by a more gradual decrease in pH$_i$ (Fig. 2D). A further decrease was observed with 5 mM dithionite, and pH$_i$ was again increased by 20 mM NH$_4^+$.

In these STIM/Orai-expressing HEK293 cells, the action of 1 mM dithionite to inhibit $I_{\text{CRAC}}$ was rapidly reversed by application of 20 mM NH$_4^+$ (Fig. 2E). The I/V curves from this experiment revealed the channel properties remained similar (Fig. 2F). The correlation between pH changes and CRAC channel function indicated that the action of dithionite was likely mediated by hypoxia-induced intracellular acidification. Although the CRAC channel measurements were undertaken with an intracellular solution buffered with 10 mM HEPES, this did not constrain intracellular acidification. When intracellular HEPES was reduced to just 1 mM, the rate of change of channel activity with dithionite was only marginally changed.

We further assessed the relationship between CRAC channel activity and pH$_i$ by directly acidifying the cytoplasm using the
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weak acid propionate. Inclusion of propionate (50 mM) in the extracellular solution reduced pH_i rapidly as a result of proton release within the cytoplasm, and the action was rapidly reversed by NH_4^+ (20 mM) (Fig. 2F). Consistently, CRAC channel activation was inhibited by application of propionate (50 mM), and this inhibition was reversed by 20 mM NH_4^+ (Fig. 2G). The I/V curves before and after propionate and NH_4^+ additions remained similar. The action of propionate to release protons within the cytoplasm resulted in a faster change in pH_i than the action of dithionite that changes pH through oxygen depletion and blockade of cellular respiration (39–41). How-

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FIGURE 3. Hypoxia does not reverse store-dependent STIM1/Orai1 junctional assembly but does induce Ca^{2+} release and STIM1 junctional accumulation. High resolution imaging of ER/PM junctions adjacent to the coverslip in HEK293 cells cotransfected with Orai1-CFP and STIM1-YFP. A–C, before treatment. D–F, same cells 5 min after 2 μM ionomycin addition. G–I, cells after a further 5-min treatment with 1 mM dithionite (Na-Dit). J and K, STIM1-YFP distribution in stable-expressing HEK cells either before (J) or 5 min after (K) 1 mM dithionite. L, Ca^{2+} release (0 external Ca^{2+}) in the same STIM1-YFP-expressing HEK cells in response to 1 mM dithionite. M and N, STIM1-YFP distribution in stable-expressing HEK cells either before (M) or 5 min after (N) 20 mM propionate. O, Ca^{2+} release (0 external Ca^{2+}) in the same STIM1-YFP-expressing HEK cells in response to 20 mM propionate.

From these results, we conclude that although hypoxic conditions actually induce depletion of stores and STIM1 translocation into junctions, the activation of Orai1 channels is in fact...
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We therefore examined how hypoxia may modify the STIM-Orai coupling process that leads to channel activation. For this, we used specific segments of the cytoplasmic domain of STIM1. Recent studies reveal that SOAR, a small fragment of the STIM1 cytoplasmic C-terminal domain, is sufficient to fully activate Orai1 channels (1, 44, 45). We expressed the SOAR domain of STIM1 (Fig. 4A), comprising residues 344–442 (44), in HEK293 cells stably expressing Orai1-CFP (23). The SOAR fragment binds tightly to Orai channels independently of store depletion (9, 44, 45). As shown in Fig. 4, B–D, binding of YFP-SOAR occurs over the entire PM and is not restricted to junctional regions. In the SOAR/Orai1 overexpressing cells, I_{CRAC} is constitutively active without store depletion (Fig. 4H). Indeed, the rounded appearance of SOAR-expressing cells reflects some Ca^{2+} overload as noted earlier (44, 45). Application of 1 mM dithionite caused a rapid and almost complete inhibition of constitutive SOAR-activated I_{CRAC} (Fig. 4H). Upon dithionite removal, there was substantial reversal of the inhibition, and Orai channel activity returned. The I-V relationship revealed the specificity and selectivity of Orai channels before and after dithionite was unchanged (Fig. 4I). However, despite complete channel inhibition, dithionite had no effect on YFP-SOAR or Orai-CFP distribution, with no visible release of SOAR from the PM (Fig. 4, E–G). Propionate also had no effect on SOAR or Orai distribution (supplemental Fig. S4). This suggested that dithionite (or propionate) is more likely changing the STIM/Orai conformational coupling process rather than altering STIM-Orai binding per se. Also, the action of dithionite is clearly independent on Ca^{2+} store depletion. Moreover, because the entire N-terminal Ca^{2+}-sensing luminal domain of STIM1 is absent in SOAR, the action of dithionite is clearly not mediated by modifying aggregation or translocation of STIM1 that is dependent on this N-terminal region (1). In addition, the absence in SOAR of the far C-terminal region, which includes the K-rich segment that assists in the association of STIM1 with the PM (45), indicates that dithionite does not work by modifying association of the C-terminal region with the PM.

We also examined the actions of hypoxia on cells expressing the complete C-terminal 235–685 cytosolic segment of STIM1 (STIM1ct) (Fig. 4A). This segment is a poor activator of Orai channels (23, 45, 46), and unlike SOAR, it remains largely free within the cytosol even in Orai1-coexpressing cells (Fig. 4L). The Orai1-interacting domain of STIM1ct is occluded through an intramolecular electrostatic interaction thought to play an important role in regulating STIM1 function (43, 46–48). The 2-APB molecule profoundly changes STIM1ct, allowing it to avidly bind to Orai1 and fully but transiently activate Ca^{2+} entry through the Orai1 channel (Fig. 4J) (23). Prior treatment of cells for 2 min with 1 mM dithionite only partially reduced the Ca^{2+} entry activated by 2-APB (Fig. 4K). In cells cotransfected with YFP-STIM1ct and Orai1-CFP (Fig. 4, L–N), 1 mM dithio-
nite for 2 min did not alter the distribution of YFP-STIM1-ct or Orai1-CFP in the cytoplasm and PM, respectively (Fig. 4, O–Q). Moreover, the 2-APB-induced association of YFP-STIM1ct with Orai1-CFP at the PM was also unaltered by dithionite (Fig. 4, R–T). Thus again, the interaction between the two proteins is insensitive to hypoxia, although the channel activation is inhibited. The lower sensitivity to hypoxia of the 2-APB-induced transient channel activation by STIM1ct (Fig. 4, J and K) suggested the activation process with 2-APB is not the same as with SOAR, as discussed below. Moreover, because the Orai channel remains at least partially activated by 2-APB in the presence of 1 mM dithionite, this provides further evidence against a direct pore blocking action of dithionite.

Although physical interaction between STIM and Orai was not prevented by hypoxia, functional coupling between the proteins to activate the channel was blocked. To understand more about the actions of dithionite on the coupling process between STIM and Orai, we examined Förster resonance energy transfer (FRET) between the C-terminally labeled STIM1-YFP and the C-terminally labeled Orai1-CFP expressed in HEK293 cells (Fig. 5). Ionomycin caused a large and rapid increase in STIM-Orai FRET consistent with the close association that occurs in the ER-PM junctions as a result of Ca\(^{2+}\) store depletion (Fig. 5A). Addition of 1 mM dithionite caused a rapid reduction in FRET almost completely reversing it within 100 s. Thereafter, application of NH\(_4^+\)/Na\(^+\) caused a rapid and transient increase in FRET. The FRET changes are consistent with the changes in pH and \(I_{\text{CRAC}}\) observed in these cells in response to dithionite and NH\(_4^+\) (Fig. 2). Controls established that the induced pH changes had only marginal direct effects on FRET measurements (see below and under “Experimental Procedures”). We also examined direct modification of pH\(_i\) by propionate on STIM-Orai FRET (Fig. 5B) and again revealed that decreased intracellular pH\(_i\) caused reduced STIM-Orai coupling that was reversed by NH\(_4^+\)-induced elevation of pH\(_i\). The action of dithionite was also observed on store depletion-induced FRET...
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between STIM2-YFP and Orai1-CFP (Fig. 5C). In this case, the dithionite-induced inhibition of STIM2-Orai1 FRET was larger than the ionomycin-induced signal reflecting the greater constitutive activation by STIM2 because of its increased sensitivity to store depletion (1, 49). In further studies, we examined constitutive FRET between YFP-SOAR and Orai1-CFP and observed a clear decrease induced by dithionite, which was rapidly reversed by NH₄⁺ addition (Fig. 5D) with kinetics similar to those of whole STIM1. The dithionite-induced STIM-Orai and SOAR-Orai FRET changes were unexpected because no dissociation of either STIM1 (Fig. 3) or SOAR (Fig. 4, B–G) was observed with dithionite. We interpret the FRET changes to indicate a conformational change in the STIM-Orai coupling complex induced by changed pH. When NH₄⁺ was added prior to dithionite (Fig. 5E), the briefly observed FRET signal was likely artificial. The strong association between SOAR and Orai1 could not be further enhanced by increasing pH. Subsequent dithionite addition caused little further FRET change as expected because NH₄⁺ prevented further change in pH. Finally, we examined the actions of dithionite and NH₄⁺ on FRET signals between the cytoplasmically expressed STIM1ct-YFP and Orai1-CFP in the PM. As described above, 2-APB induces a strong association between STIM1ct and Orai1, and this interaction is detected as a substantial 2-APB-induced increase in FRET between STIM1ct-YFP and Orai1-CFP (Fig. 5F). Interestingly, addition of dithionite or NH₄⁺ did not significantly change the STIM1ct-YFP/Orai1-CFP FRET signal, and thus, the avid 2-APB-induced interaction between STIM1ct and Orai1 is unaffected by pH. This suggests that 2-APB is able to lock the STIM1ct and Orai1 molecules into a pH-insensitive interaction, as discussed below. This result also shows that pH changes do not directly alter the FRET signal.

DISCUSSION

The Ca²⁺ store-sensing STIM proteins are established mediators of Ca²⁺ entry signals and Ca²⁺ homeostasis (1, 7, 43). Recent information indicates that STIM protein activation (i.e. STIM1 aggregation and translocation into junctions) occurs in response to distinct cellular stress conditions. Sustained reduction of Ca²⁺ within the lumen of ER is a major cellular stress condition in cells, resulting in misfolding of proteins (50) and activation of the unfolded protein response to mitigate cellular damage (51). More typically, receptors cause transient release of Ca²⁺ from limited ER stores, and the Ca²⁺ entering through Orai channels contributes to the generation of cytosolic Ca²⁺ signals that turn on longer term cellular responses, including transcription and cell growth (6, 7). Oxidative stress represents another major stress condition, and STIM1 can be activated through cysteine modification induced by reactive oxygen species (11). In addition, heat stress induces activation of STIM1, with the small increases in temperature causing STIM1 to aggregate and translocate into junctions (12, 13).

The results here reveal that STIM protein activation and STIM-Orai coupling are key mechanisms in cellular responses to yet further stress conditions, hypoxia and acidosis. We reveal that hypoxia itself can cause activation of STIM1 likely as a result of lowered ATP levels and decreased Ca²⁺ pumping into stores. Such Ca²⁺ store depletion would ordinarily lead to Orai channel activation and Ca²⁺ entry. However, the ensuing pH decrease has a powerful attenuating effect on the functional coupling between STIM and Orai that gates channel opening. This action may function as an important protective event to prevent hypoxia-induced Ca²⁺ overload. Hence, with a reduced ability to pump Ca²⁺ out of the cytosol during hypoxia, the constitutive entry of Ca²⁺ through Orai channels would have severe consequences for cellular function. This compensatory control mechanism has some similarities to the responses of STIM and Orai to temperature (13). Thus, increased temperature causes STIM activation (aggregation and junctional translocation) but at the same time inhibits the coupling to activate Orai channels. It is possible that this is a similar compensatory process to protect cells at higher temperatures from Ca²⁺ overload.

Two recent studies published after completion of this paper revealed that hypoxia can cause store depletion through production of low levels of ROS as opposed to decreased ATP levels (52, 53). The resulting store depletion caused activation of SOCE, although this was not characterized as being mediated by a CRAC current. Our studies are in agreement with this result because we also observed hypoxia-induced store depletion and STIM1 activation. However, in our case, the ensuing Ca²⁺ entry is attenuated by hypoxia-induced acidification. Our focus has been to examine the actions of hypoxia and ensuing pH changes after store depletion-induced CRAC channel activation, i.e. after coupling between STIM and Orai had been established. In another study, it was reported that ROS may have direct inhibitory actions on Orai channels (54). Our results reveal that hypoxia induces acidification, and that direct induction of acidification with propionate mimics the action of hypoxia. These results together suggest pH change is responsible for Orai channel inhibition and mitigate against the action of hypoxia being mediated by ROS.

The effects of pH on STIM-Orai coupling are compatible with recent studies that reveal that STIM-Orai association involves electrostatic interactions between basic amino acids in the SOAR region of STIM1 and acidic residues on the cytoplasmic C-terminal tail of Orai1 (43, 46–48). These electrostatic interactions are crucial to the functional coupling between activated STIM proteins and Orai channels that leads to “signal transmission” and gating of the Orai channel (43, 48). We hypothesize that increased proton levels induce dissociation of the electrostatic interactions that are required for Orai channel activation; hence the channel closes. However, despite this uncoupling of channel activation, we do not observe any obvious dissociation between either STIM or Orai or between SOAR and Orai under the conditions of hypoxia and decreased pH. Yet under the same conditions we do observe clearly identifiable decreases in FRET signals between STIM and Orai and between SOAR and Orai. This apparent contradiction may be explained by recent information that the cytoplasmic domain of STIM1 contains two sites of interaction with Orai1 (43, 45, 46, 48), and these are in addition to a strong interaction of the far C-terminal polybasic region with the PM (see Fig. 6) (1, 45). The two Orai-interacting sites in STIM1 are both retained within the short SOAR/CAD domain (45). Thus, in addition to the electrostatic interaction with the Orai1 C terminus that is
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FIGURE 6. Model to depict actions of hypoxia-induced acidosis on STIM1-Orai1 coupling. A, resting state. Ca\(^{2+}\) stores are full; STIM1 is inactivated and uncoupled from Orai1 channels. B, activated state. Stores are depleted of Ca\(^{2+}\), and STIM1 is activated and coupled to opened Orai1 channels through the electrostatic STIM-Orai coupling site (A) between SOAR and the C terminus of Orai1. A second site between SOAR and the Orai1 N terminus also exists (B); the STIM1 C-terminal polylysine region is bound to PM lipids. C, acidosis-induced uncoupling. Lowered pH causes dissociation of the electrostatic STIM1-Orai1 coupling and Orai1 channels close; STIM1 remains attached to Orai1 through interactions between SOAR and the N-terminal Orai-binding site; the STIM1 C-terminal polylysine region remains attached to the PM.

crucial for signal transmission and channel gating. STIM1 has a separate interaction within the Orai1 N-terminal domain (45). As shown in Fig. 6, we speculate that the decreased FRET signal arises from a pH-induced dissociation of the electrostatic interaction site (and concomitant channel closing) leading to a FRET-sensitive conformational change, whereas the other interaction site remains intact preventing complete dissociation between STIM1 and Orai1 (Fig. 6C). Such a two-site interaction model was recently suggested by Calloway et al. (48).

These findings provide a new mechanistic link between hypoxia and Ca\(^{2+}\) signal generation. They reveal that the STIM-Orai signaling complex senses and responds to hypoxic stress and that the ensuing acidification-mediated functional uncoupling of the STIM-Orai complex is an important mechanism for protecting cells from Ca\(^{2+}\) overload under hypoxic stress conditions. Such mechanisms may be crucial in understanding both acute and long term pathophysiological responses to hypoxia and ischemia, for example the hypoxia-induced modification of airway and vascular tone or the damaging Ca\(^{2+}\) overload occurring during ischemia reperfusion of tissues. Understanding the mechanisms and identifying the molecular targets that mediate hypoxic responses have great significance toward not only major vascular and pulmonary disease states but also toward cancer in which hypoxic conditions contribute greatly to altered cell function.

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