Hypoxia Produces Pro-arrhythmic Late Sodium Current in Cardiac Myocytes by SUMOylation of Na\textsubscript{V}1.5 Channels

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SUMMARY

Acute cardiac hypoxia produces life-threatening elevations in late sodium current (\(I\textsubscript{LATE}\)) in the human heart. Here, we show the underlying mechanism: hypoxia induces rapid SUMOylation of Na\textsubscript{V}1.5 channels so they reopen when normally inactive, late in the action potential. Na\textsubscript{V}1.5 is SUMOylated only on lysine 442, and the mutation of that residue, or application of a deSUMOylating enzyme, prevents hypoxic re-openings. The time course of SUMOylation of single channels in response to hypoxia coincides with the increase in \(I\textsubscript{LATE}\), a reaction that is complete in under 100 s. In human cardiac myocytes derived from pluripotent stem cells, hypoxia-induced \(I\textsubscript{LATE}\) is confirmed to be SUMO-dependent and to produce action potential prolongation, the pro-arrhythmic change observed in patients.

INTRODUCTION

In the human heart, Na\textsubscript{V}1.5 voltage-gated sodium channels pass \(I\textsubscript{Na}\), a rapidly activating and inactivating Na\textsuperscript{+} current that determines the rise, duration, and timing of action potentials (Marbán, 2002; Catterall, 1986). In addition, Na\textsubscript{V}1.5 channels pass a small, persistent Na\textsuperscript{+} current, the late sodium current (\(I\textsubscript{LATE}\)), which contributes to maintaining the action potential plateau (Makielski, 2016). In healthy hearts, \(I\textsubscript{LATE}\) is less than 0.5% of the magnitude of peak \(I\textsubscript{Na}\) and is passed by a small number of channels that do not stay closed after inactivation (Makielski, 2016). In patients with an ischemic and failing heart, sudden infant death syndrome (SIDS), and mutations in SCN5A, the gene that encodes the Na\textsubscript{V}1.5 channel, which produces long QT syndrome, the size of \(I\textsubscript{LATE}\) can increase to 4%–5% of peak \(I\textsubscript{Na}\) (Bennett et al., 1995; Plant et al., 2006; Belardinelli et al., 2015). Furthermore, acute hypoxia and ischemia have been recognized to increase \(I\textsubscript{LATE}\) in cardiac myocytes (Saint et al., 1992; Ju et al., 1996; Carmeliet, 1999; Belardinelli et al., 2006) prior to slower processes like remodeling (West, 2017), and excess \(I\textsubscript{LATE}\) has been shown to be pro-arrhythmic because it prolongs action potential duration (APD), reducing repolarization reserve, increasing susceptibility to after-depolarizations, and causing a predisposition to torsades de pointes (TdP), a ventricular dysrhythmia that is lethal when sustained (Gaur et al., 2009; Shryock et al., 2013; Chadda et al., 2017). Thus, increases in \(I\textsubscript{LATE}\) above baseline by just 0.3% to 1% predisposition to sudden cardiac death (Bennett et al., 1995; Belardinelli et al., 2015). These observations underpin proposals that inhibiting \(I\textsubscript{LATE}\) has therapeutic potential (Belardinelli et al., 2006; Song et al., 2006).

We recently reported that the rapid influx of Na\textsuperscript{+} flux into neurons in response to acute hypoxic challenge is due to SUMOylation of Na\textsubscript{V}1.2, the major voltage-gated sodium channel in the brain; in that channel, SUMO modification of lysine 38 alters opening and closing with changes in voltage and does not effect \(I\textsubscript{LATE}\) (Plant et al., 2016). SUMOylation is the enzyme-mediated linkage of one of three SUMO isoforms to the ε amino group of specific lysine residues in a target protein (Henley et al., 2014). Present in all eukaryotic cells, the SUMO pathway was known to regulate the trafficking and activity of nuclear transcription factors when we described it to operate as well at the plasma membrane by direct SUMOylation of Na\textsuperscript{+} and K\textsuperscript{+} channels to regulate excitability (Rajan et al., 2005; Plant et al., 2010, 2011, 2012, 2016; Xiong et al., 2017). Given the important role of hypoxia-induced increases in \(I\textsubscript{LATE}\) in heart disease, we tested the hypothesis that SUMOylation of Na\textsubscript{V}1.5 channels was the underlying mechanism.

We demonstrate that hypoxia induces a rapid increase in \(I\textsubscript{LATE}\) in human cardiac myocytes derived from pluripotent stem cells (iPS-CMs). The response is reproduced by application of cytosolic SUMO1 at ambient levels of oxygen and suppressed by the deSUMOylating enzyme SENP1. SUMOylation of Na\textsubscript{V}1.5 on lysine 442, a residue located in the segment between channel domains I and II, is shown to be necessary and sufficient to explain hypoxia-induced changes in \(I\textsubscript{LATE}\) by reconstitution of the response in heterologous cells and by studies using whole-cell and single-channel patch-clamp recording and live cell Förster resonance energy transfer (FRET). The implied mechanism was confirmed using total internal reflection fluorescence microscopy (TIRFM) to study single particles on the surface of live cells in real-time; the time course was the same for hypoxic challenge, the recruitment of SUMO1 to Na\textsubscript{V}1.5 channels on cell surface, and the increase in \(I\textsubscript{LATE}\). Furthermore, hypoxia-induced, SUMO-dependent increases in \(I\textsubscript{LATE}\) were observed to increase APD.
in human induced pluripotent stem cells (iPSCs) by recording spontaneous action potentials in current-clamp mode; and the changes were suppressed by ranolazine, a drug that inhibits NaV1.5 channel late current. Incorporating the measured hypoxia-induced increase in \( I_{\text{LATE}} \) in IPS-CMs into the O’Hara-Rudy model for human cardiac action potentials (O’Hara et al., 2011) was sufficient to reproduce the observed pro-arrhythmic increase in APD. Given the role of \( I_{\text{LATE}} \) in arrhythmogenesis, SUMOylation of NaV1.5 is identified as a mechanistically defined target for therapeutic intervention.

RESULTS

HYPOXIA RAPIDLY INCREASES \( I_{\text{LATE}} \) IN HUMAN IPS-CMS

At ambient \( O_2 \) levels (21%), \( I_{\text{Na}} \) in human IPS-CMs activated rapidly to a mean peak of \(-40 \pm 4 \) pA/pF when measured at \(-30 \) mV in whole-cell mode (Figure 1A; Table 1). \( I_{\text{Na}} \) then inactivated to produce a residual \( I_{\text{LATE}} \) that was 0.46% ± 0.1% of the magnitude of peak current 50 to 100 ms after the onset of inactivation. When \( O_2 \) was lowered from ambient levels to 1.5% by the perfusion of cells with a hypoxic solution (Plant et al., 2016), mean peak \( I_{\text{Na}} \) was unchanged (\(-42 \pm 3 \) pA/pF), but \( I_{\text{LATE}} \) increased 

SUMO1 INCREASES \( I_{\text{LATE}} \) IN HUMAN IPS-CMS

We delivered 1 nM purified SUMO1 peptide into iPS-CMs by the patch pipette; a concentration that evoked maximal effects on NaV1.2 channels in cerebellar granule neurons (CGNs) and three different K’ channels in CGNs, hippocampal neurons, and rat cardiac myocytes (Xiong et al., 2017; Plant et al., 2011, 2012, 2016). SUMO1 induced changes in cardiac \( I_{\text{Na}} \) like those produced by hypoxia: \( I_{\text{LATE}} \) increased to \(-4.2\% \) of peak \( I_{\text{Na}} \) without a change in the peak magnitude (Figures 1B and 1C; Table 1). When SUMO1 was in the recording pipette, changing the solution bathing IPS-CMs from 21% to 1.5% \( O_2 \) did not further alter the parameters of \( I_{\text{Na}} \) peak or \( I_{\text{LATE}} \). The notion that hypoxic regulation of \( I_{\text{Na}} \) proceeds by SUMOylation was further supported by the observation that delivering 1 nM SENP1 deSUMOylases into the IPS-CMs by the recording pipette suppressed the increase in \( I_{\text{LATE}} \) induced by hypoxia (Figures 1B and 1C; Table 1).

\( I_{\text{LATE}} \) is observed in the “window” between the voltages where Na\( \lambda \) channels activate and inactivate (Figure S1; Table 1). Thus, exposure of the IPS-CMs to 1.5% \( O_2 \) caused a leftward shift in the half-maximal activation voltage of \( I_{\text{Na}} \) (\( V_{1/2-\text{act}} \)) of \(-6 \pm 4 \) mV and a rightward shift in the steady-state inactivation midpoint (\( V_{1/2-\text{SSI}} \)) of 6 ± 3 mV, increasing the range of voltages over which \( I_{\text{Na}} \) channels are likely to be activated but not fully inactivated. In ambient \( O_2 \), SUMO1 in the pipette increased the magnitude of the window current in a manner like that observed on exposure to 1.5% \( O_2 \), and hypoxic challenge with SUMO1 in the pipette did not further alter the parameters. Furthermore, inclusion of 1 nM SENP1 prevented the effects of hypoxia, maintaining the window current at control levels.
### Table 1. Effects of Hypoxia, SUMO1, and SENP1 on \( I_{\text{Na}} \) in iPS-CMs and NaV1.5 Channels in CHO Cells

| Condition | Activation | \( \Delta \text{pA/pF} \) | Current | \( \Delta \text{pA/pF} \) | \( \Delta \text{pA/pF} \) |
|-----------|------------|-----------------|---------|-----------------|-----------------|
| \( O_2 \)  | 21\%       | 1.5\%           | 1.5\%   | 2\%             | 2\%             |
| SUMO1     | 44.6 ± 2   | 42 ± 2          | 42 ± 2  | 0.28 ± 0.7      | 0 ± 0.6         |
| SENP1     | 44.6 ± 2   | 42 ± 2          | 42 ± 2  | 0.28 ± 0.7      | 0 ± 0.6         |
| SUMO1     | 44.6 ± 2   | 42 ± 2          | 42 ± 2  | 0.28 ± 0.7      | 0 ± 0.6         |
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Of note, the kinetics of recovery of \( I_{\text{Na}} \) from the fast-inactivated state was insensitive to hypoxia, SUMO1, and SENP1 (Figure S1).

### SUMOylation of Na\(_v\)1.5 on Lys442 Is Necessary and Sufficient to Recapitulate the Hypoxic Response

In cardiomyocytes, Na\(_v\)1.5 channel complexes contain the 2016 amino-acid-pore-forming subunit (Figure 2A) and an accessory subunit, Na\(_v\)β1 (Catterall, 2012). A combination of spectroscopic and electrophysiological assays in live CHO cells showed that SUMOylation in response to hypoxia occurred only on Na\(_v\)1.5 K442 and that modification of the site mediated the response to acute hypoxia.

First, the association of heterologously expressed human Na\(_v\)1.5 and SUMO proteins at the surface of cells was studied by measuring FRET between subunits tagged with the fluorescent proteins mTFP1 and YFP (Figure 2A). The Na\(_v\)β1 subunit was expressed in these studies but did not carry a fluorescent protein. Tagging the N terminus of Na\(_v\)1.5 (mTFP-Na\(_v\)1.5) did not alter the biophysical properties of the channel nor its responsiveness to hypoxia or SUMO1 or SENP1 in the recording pipette (Figures S2; Table S1). Similarly, tagging the N terminus of SUMO1 (YFP-SUMO1) did not alter its operation with Na\(_v\)1.5, as previously observed with Na\(_v\)1.2 and three K\(^+\) channels (Xiong et al., 2017; Plant et al., 2010, 2011, 2012, 2016).

Both mTFP1-3Na\(_v\)1.5 and YFP-SUMO1 were visualized at the plasma membrane when expressed in CHO cells (Figure 2A). Indicating FRET, and thus the intimate association of the two fluorophores consistent with SUMOylation, the time course of mTFP1 photobleaching under continuous illumination had a time constant (\( \tau \)) that was prolonged from less than 10 s to 27 s when the channel was expressed with SUMO-SUMO1 (Figures 2B and 2C). mTFP-Na\(_v\)1.5 also showed FRET with YFP-Ubc9, the SUMO conjugating enzyme that binds to target proteins only when a lysine subject to modification is present (Xiong et al., 2017; Plant et al., 2016). In contrast, FRET was not observed when mTFP-Na\(_v\)1.5 was expressed with linkage-incompetent YFP-SUMO1\(_{95}\) (a variant that lacks the terminal Gly-Gly motif present in SUMO1 and the matured isoform SUMO1\(_{97}\)) or soluble YFP; in both cases, \( \tau \) was less than 10 s.

To identify SUMOylation site(s) in Na\(_v\)1.5 channels, we analyzed the primary sequence of the subunit with an algorithm that looks for variants of the canonical SUMO motif \( \psi-K-X-E/D \), as before with Na\(_v\)1.2 and the K\(^+\) channels (Xiong et al., 2017; Plant et al., 2010, 2011, 2012, 2016). We mutated each of five potential target lysines individually to glutamine in mTFP-Na\(_v\)1.5 and used FRET with YFP-SUMO1 to assess interaction (Figure S2). Only K442, within the motif L-K\(_{442}\)-K, was required to produce FRET. Thus, mTFP-Na\(_v\)1.5-K442Q was expressed on the cell surface like wild-type mTFP1-Na\(_v\)1.5 but did not show FRET with YFP-SUMO1 or YFP-Ubc9 (Figure 2C). The absence of FRET supported the conclusion that K442 was necessary for SUMOylation of the Na\(_v\)1.5 channel complex and, moreover, that no other lysine residue in the \( \alpha \) subunit or in the Na\(_v\)β1 was subject to SUMOylation.
To confirm that K442 in the Na\textsubscript{v}1.5 sequence motif was subject to SUMOylation, a mass spectrometry (MS) strategy was used that we had previously developed for K\textsuperscript{+} channels (Plant et al., 2010, 2011). SUMO1\textsubscript{97}T95K, a variant that leaves a Gly-Gly remnant of SUMO1 on the \textepsilon amino group of the target Lys after enzyme cleavage, was expressed with Na\textsubscript{v}1.5 residues 353–502 bearing a His\textsubscript{6} affinity tag and the mammalian E1 and E2 SUMOylation enzymes in bacteria. Affinity purification followed by SDS-PAGE revealed a band of the expected apparent mass for the Na\textsubscript{v}1.5-SUMO adduct (∼35 kDa). The purified protein was treated with trypsin and subjected to MS to yield a sequence coverage for Na\textsubscript{v}1.5\textsubscript{353–502} of 42% and was identified uniquely as the fragment carrying the Gly-Gly remnant (Figure S4).

Next, we confirmed that K442 controlled the response to hypoxia by studying human Na\textsubscript{v}1.5 channels expressed in CHO cells. With the wild-type channel, decreasing O\textsubscript{2} from 21% to 1.5% was associated with a rapid increase in \( I_{\text{LATE}} \) from ∼0.5% (−0.96 ± 0.2 pA/pF) to −4.1% (−8.3 ± 0.6 pA/pF), without a change in the peak current, −200 pA/pF at −30 mV (Figure 3A; Table 1), as observed for \( I_{\text{LATE}} \) in IPS-CMs. As predicted, Na\textsubscript{v}1.5-K442Q channels were insensitive to hypoxia; \( I_{\text{LATE}} \) for the point mutant channels remained less than ∼0.5% of the peak current when O\textsubscript{2} was decreased to 1.5%. Furthermore, peak current measured at −30 mV was like wild-type channels (∼−200 pA/pF) and was also unaltered by hypoxia (Figures 3A and 3B). Of note, the time course for the change in \( I_{\text{LATE}} \) for Na\textsubscript{v}1.5 channels heterologously expressed in the CHO cells was like that for native \( I_{\text{Na}} \) in IPS-CMs, increasing from 0.5 to 4.2% over ∼100 s, with half the rise time of 39.7 ± 2 s, and the increase was stable after the cells were restored to ambient levels of O\textsubscript{2} for at least 3 min (Figure 3C).

Na\textsubscript{v}1.5 channels heterologously expressed in tissue culture cells also responded to SUMO1 and SENP1 like \( I_{\text{Na}} \) in IPS-CMs. Thus, Na\textsubscript{v}1.5 channels with SUMO1 in the recording pipette showed \( I_{\text{LATE}} \) of ∼4% of the peak current at both 21% and 1.5% O\textsubscript{2} (Figure 3C; Table 1). Furthermore, the window-current voltages increased in 1.5% O\textsubscript{2} with 1 nM SUMO1, by −6 mV for \( V_{1/2}\text{–act} \) and +6 mV for \( V_{1/2}\text{–SSI} \) (Figure S3), as observed for \( I_{\text{Na}} \) in IPS-CMs. Also like \( I_{\text{Na}} \) in IPS-CMs, \( I_{\text{LATE}} \) for Na\textsubscript{v}1.5 channels was below 0.5%, with 1 nM SENP1 in the pipette at both 21% and 1.5% O\textsubscript{2}, with no change in the window current, \( V_{1/2}\text{–act} \), or \( V_{1/2}\text{–SSI} \). Finally, the biophysical properties of Na\textsubscript{v}1.5-K442Q channels were insensitive to hypoxia and...
were not altered by SUMO1 or SENP1, showing less than 0.5% $I_{LATE}$ and without changes in $V_{1/2}$-act and $V_{1/2}$-SSI.

**Single NaV1.5 Channels Are Reopened from the Inactive State by Hypoxia**

Single NaV1.5 channels are closed at rest, open briefly upon depolarization, and then inactivate rapidly; the channels remain in the inactive state until the membrane returns to the resting potential and the channels move back to the closed state from which they are available to open once again in response to the next heartbeat. Thus, single NaV1.5 channels in cell-attached patches open just once in response to depolarization and almost always remain inactive (Figure 3D). As predicted by their behavior in whole-cell mode, hypoxia induced abnormal single-channel reopenings during sustained depolarization. Ensemble currents generated by the cumulative averaging of 300 repetitive cycles of depolarization demonstrated that the reopening of inactivated NaV1.5 channels in response to hypoxia was sufficient to increase $I_{LATE}$ from 0.4% ± 0.1% to 4.8% ± 0.3%. In contrast, NaV1.5-K442Q channels opened only once and remained inactive when cells were studied in either ambient levels of O$_2$ or at 1.5% O$_2$ (Figure 3E).

**Single NaV1.5 Channels Are Reopened from the Inactive State by SUMOylation**

To confirm the mechanistic role of SUMO, single NaV1.5 channels were studied in inside-out membrane patches excised from CHO cells, allowing perfusion of the intracellular face of...
Figure 4. Hypoxia Recruits One SUMO1 Monomer to Each Cell Surface Na\textsubscript{v}1.5 Channel

Single mTFP-Na\textsubscript{v}1.5 channels (blue) and SUMO1 tagged with mCherry (m-SUMO1, red) were studied in CHO-K1 cells by TIRFM. Stoichiometric (photobleaching) and pixel-by-pixel analysis for subunit density and co-localization were performed as described in the STAR Methods. Manders’ coefficients were assessed post hoc for 3–5 regions per cell, and co-localization was defined as the presence of both fluorophores at more than 30% of the maximum fluorescence level recorded in that stack (and their overlap is represented in the images as white pixels). The time course of hypoxic modulation of Na\textsubscript{v}1.5 \textit{ILATE} was studied with steps from –100 mV to /C\textsubscript{0} 30 mV every 10 s and normalized to the peak current. Data represent 5–8 cells, and biophysical parameters and single particle statistical analyses are summarized in Tables S1 and S3.

(A) Left: single co-localized particles with mCherry and TFP fluorescence were observed at the surface of cells expressing Na\textsubscript{v}1.5 and SUMO1. The time courses for simultaneous photobleaching of the fluorophores revealed that complexes have one subunit of each type.

(B) Histogram of photobleaching steps showing that hypoxia increased single m-SUMO1 (red) subunits at the cell surface co-localized with mTFP-Na\textsubscript{v}1.5 channels (blue), without a change in subunit stoichiometry.

(legend continued on next page)
the channel with experimental solutions. In ambient O₂, single Naᵥ1.5 channels opened once and remained inactive for the duration of the depolarizing step, and in this configuration reducing O₂ from 21% to 1.5% did not produce channel reopening (Figure S5). This suggested that hypoxia-induced reopening in whole-cell mode depended on the presence of cytoplasmic SUMO, a hypothesis confirmed when patches were perfused with 1 nM SUMO1, and reopening was observed at both 21% and 1.5% O₂.

Like the changes observed in whole-cell mode with hypoxia, single channels in the patches reopened within 150 s of exposure to SUMO1, with 50% of the patches showing channel reopening at 65 ± 10 s (Figure S5). In agreement with the cell-attached recordings, ensemble currents generated from single Naᵥ1.5 channels by averaging 500 cycles of depolarization in off-cell patches showed that SUMO1 increased Iₐ₀ from 0.4% ± 0.2% to 4.1% ± 0.3% (Figure S5). As anticipated, single Naᵥ1.5-K442Q channels in inside-out patches opened once and did not reopen even when exposed to SUMO1 or hypoxia (Figure S5). When considered in conjunction with the cell-attached studies, these findings support three conclusions: SUMOylation is necessary and sufficient for aberrant reopening of single Naᵥ1.5 channels, K442 is required to mediate the effect of SUMO1 on Naᵥ1.5, and hypoxia-evoked changes in Iₐ₀ are mediated by the SUMO pathway.

**Hypoxia Induces Rapid monoSUMOylation of Naᵥ1.5 Channels**

To determine the stoichiometry of SUMOylation, we directly counted the number of SUMO1 monomers on individual Naᵥ1.5 channels at the surface of live CHO cells by using TIRFM photobleaching, as before for Naᵥ1.2 and IKs channels (Xiong et al., 2017; Plant et al., 2014, 2016). When mTFP-Naᵥ1.5 and SUMO1 tagged with mCherry (m-SUMO1) were co-expressed, particles containing only mTFP-Naᵥ1.5 and others with both mTFP-Naᵥ1.5 and m-SUMO1 were observed at the cell membrane (Figures 4A and 4B; Table 2). Approximately 10% of mTFP-Naᵥ1.5 channels were co-localized with one m-SUMO1 in ambient oxygen: continuous, two-color excitation bleached each fluorophore in the complexes in a single step consistent with one subunit of each type. On exposure to hypoxia, m-SUMO1 was recruited to the cell surface but only at sites with mTFP-Naᵥ1.5, increasing the number of pixels that contained both mTFP-Naᵥ1.5 and m-SUMO1 channels from 10% to 87% within ~100 s (Figure 4B). Increased co-localization did not alter the 1:1 stoichiometry of Naᵥ1.5 channel and m-SUMO1 complexes, as judged by bleaching.

Evidence that the co-localization represented a covalent interaction of the channel and SUMO1 was provided by two additional experiments (Figure S6). First, m-SUMO1 was not observed at the membrane in cells expressing mTFP-Naᵥ1.5-K442Q and rarely yielded co-localization of the fluorophores in 21% or 1.5% O₂. Second, hypoxia did not increase the number of particles at the membrane with m-SUMO1, the SUMO1 variant that is unable to covalently attach to target lysines. Because Naᵥ1.5 channels contain just one α-subunit, the observed 1:1 stoichiometry with SUMO1 supports three additional conclusions: Naᵥ1.5 subunits are SUMOylated only on K442, the channel is monoSUMOylated and polySUMO1 chains were not observed, and the Naᵥβ1 subunit is not SUMOylated.

Although hypoxia recruited m-SUMO1 to the cell surface, it did not change the number of Naᵥ1.5 channels at the plasma membrane (Figures 4C and 4D; Table 2). This was shown by studying the surface density and localization of mTFP-Naᵥ1.5 and m-SUMO1 subunits by using pixel-by-pixel analysis, a method we have applied before (Xiong et al., 2017; Plant et al., 2014, 2016). In ambient O₂, about 10% of mTFP-Naᵥ1.5 fluorescent pixels were localized with m-SUMO1 (33 ± 3 pixels/μm²) and 90% of the channels were unmodified (305 ± 21 pixels/μm²). After 100 s of hypoxia, the ratio was reversed; the number of mTFP-Naᵥ1.5 pixels co-localized with m-SUMO1 increased to about 90% (301 ± 13 pixels/μm²), and 10% of the channels remained unmodified by m-SUMO1 (37 ± 4 pixels/μm²). Thus, hypoxia increased the surface density of m-SUMO1 ~33-fold (from 10/pixels/μm² to 334/pixels/μm²), whereas the density of mTFP-Naᵥ1.5 did not change (338/pixels/μm²).

Consistent with cause and effect, the appearance of m-SUMO1 at the plasma membrane with mTFP-Naᵥ1.5, demonstrated above to require covalent modification of the channel on K442, matched the time course of hypoxia-induced increase in Iₐ₀ observed in iPS-CMs and Naᵥ1.5 channels studied in CHO cells (Figures 1 and 3; Table 1). These rates also matched the increase in the mean Manders’ coefficient of co-localization of m-SUMO1 and mTFP-Naᵥ1.5 (Figure 4E) as well as Pearson’s correlation coefficient (Table S2). Also, as observed for Iₐ₀ in iPS-CMs and Naᵥ1.5 channels in CHO cells, the number of mTFP-Naᵥ1.5 surface particles and the fraction of channels localized with m-SUMO1 were stable on restoration to ambient O₂ for 3 min after hypoxia (Figure 4E; Table 2). Supporting the notion that heterologous expression of m-SUMO1 increased basal SUMOylation of mTFP-Naᵥ1.5, about 10% of mTFP-Naᵥ1.5 channels co-localized with m-SUMO1 in these cells and Iₐ₀ was 2.5-fold higher (~1% of peak current) than the control in ambient O₂, rising to 4.5% with hypoxia (Tables 1 and S1).

(C) Left: the images show that in ambient O₂, the surface density of m-SUMO1 (top) is low compared to Naᵥ1.5 (middle) with little co-localization (bottom). Right: hypoxia recruits m-SUMO1 to the cell surface within ~100 s (top), where it is co-localized with Naᵥ1.5 channels (bottom); surface levels of Naᵥ1.5 were not observed to change (middle). The scale bar represents 10 μm.

(D) Histogram of surface density summarizing six cells studied as described in (A). In ambient O₂, the density of pixels per μm² with SUMO1 alone (red) was 3 ± 1, and 305 ± 21 for Naᵥ1.5 (blue). The density of pixels with both fluorophores (green) was 33 ± 3 per μm². Hypoxia increased co-localization to 301 ± 13 pixels per μm² and decreased the density of free Naᵥ1.5 channels (37 ± 4) without altering the density of free SUMO1 (6 ± 2).

(E) The time course for the hypoxia-induced increase in the co-localization of mTFP-Naᵥ1.5 and m-SUMO1 (Manders’ coefficient, black circle) and the magnitude of Iₐ₀, as a percentage of the peak current (open circle) were coincident. A mean Manders’ coefficient of 0.10 ± 0.01 measured in ambient O₂ increased to 0.91 ± 0.03 in ~100 s of exposure to hypoxia. The mean Iₐ₀ rose from 0.46% ± 0.1% to 4.4% ± 0.8%. Increases in the Manders’ coefficient and late current versus peak current ratio were unchanged for at least 3 min after cells were restored to ambient O₂.
**Table 2. Co-localization of SUMO1 with Na\(_v\),1.5 in Response to Hypoxia**

| Subunits Expressed | mTFP-Na\(_v\),1.5 + m-SUMO1 | mTFP-Na\(_v\),1.5-K442Q + m-SUMO1 |
|--------------------|-----------------------------|----------------------------------|
| Condition          | Ambient \(O_2\) | Hypoxia 50 s | Hypoxia 100 s | Recovery 150 s | Ambient \(O_2\) | Hypoxia 50 s | Hypoxia 100 s | Recovery 150 s |
| Single particle stoichiometry | 1:1 | 1:1 | 1:1 | 1:1 | 0:1 | 0:1 | 0:1 | 0:1 |
| SUMO1: Na\(_v\),1.5 | | | | | | | | |
| Free mTFP-Na\(_v\),1.5, pixels/\(\mu m^2\) | 305 ± 21 | 166 ± 18* | 37 ± 4** | 39 ± 5** | 328 ± 26 | 333 ± 29 | 326 ± 25 | 308 ± 19 |
| Free m-SUMO1, pixels/\(\mu m^2\) | 3 ± 1 | 4 ± 2 | 7 ± 2 | 5 ± 2 | 3 ± 1 | 4 ± 1 | 4 ± 2 | 6 ± 2 |
| Co-localized pixels/\(\mu m^2\) | 33 ± 3 | 179 ± 15** | 301 ± 13** | 302 ± 15** | 4 ± 1 | 4 ± 2 | 3 ± 1 | 4 ± 1 |

Na\(_v\),1.5 or Na\(_v\),1.5-K442Q subunits tagged with mTFP1 were expressed in CHO-K1 cells with mCherry-SUMO1 (m-SUMO1) and studied by TIRFM and whole-cell patch clamp (Figures 3 and 5). The number of photobleaching steps observed for each fluorophore in each single fluorescent spot reports the stoichiometry of the channel complex. Na\(_v\),1.5 channels are monomers and show no more than one bleaching step when tagged with mTFP1 (Figure 5). No more than one bleaching step was observed for mCherry-tagged SUMO1 subunits (free or co-localized with the channel). A 1:1 stoichiometry is maintained when cells are exposed to hypoxia (1.5% \(O_2\)). SUMO1 was not observed to co-localize with Na\(_v\),1.5-K442Q channels. The surface density of subunits was quantified as the mean of four 100- by 100-pixel regions for 6–10 cells per group. Exposure to hypoxia increased the number of SUMO1 monomers observed at the cell surface within 100 s, and almost all were co-localized with Na\(_v\),1.5. Data are mean ± SEM for 5 to 8 cells per group; * \(p < 0.05\), ** \(p < 0.01\) compared with cells studied in ambient \(O_2\) for each channel type.

**Hypoxia-Induced \(I_{LATE}\) in iPSCs Yields SUMO-Dependent APD Prolongation**

To evaluate if the increase in \(I_{LATE}\) we observed was sufficient to produce pro-arrhythmic APD prolongation, iPSC-CMs were studied in current-clamp mode and the time to repolarize the membrane to resting values during spontaneous action potentials was measured. Under control conditions, the cells had a resting potential of −45 ± 3 mV, and the phase 1 action potential upstroke mediated by \(I_{Na}\) peaked at 43 ± 4 mV (n = 6). Hypoxia increased the time for 50% restoration (APD\(_{50}\)) by 22% without changing the resting potential or the action potential height (Figure 5A). To confirm that action potential prolongation was due to an increase in \(I_{LATE}\), we applied ranolazine, an open-state blocker of Na\(_v\),1.5 channels in clinical use that preferentially inhibits \(I_{LATE}\) rather than peak \(I_{Na}\) (Maier and Sossalla, 2013). Ranolazine reversed hypoxia-induced lengthening of APD\(_{50}\) to within 5% of control values without altering other action potential biophysical parameters (Table S3).

To verify that SUMOylation mediates hypoxia-induced APD prolongation, 1 nM SUMO1 or SENP1 was included in the recording pipette. As expected, SUMO1 lengthened APD\(_{50}\) to 123% of control values without causing a significant shift in the resting membrane potential or the height of spontaneous action potentials in 21% \(O_2\), and hypoxia evoked only a further 5% increase in the APD\(_{50}\) (Figure 5B). Subsequent application of ranolazine reversed the effects of SUMOylation as it had the effects of hypoxia with control solution in the pipette, reducing APD\(_{50}\) to within 5% of its control value. In contrast, 1 nM SENP1 in the recording pipette yielded APD\(_{50}\), resting membrane potential, and action potential height like control cells studied in 21% \(O_2\), and neither hypoxia nor ranolazine altered the three parameters of excitability (Figure 5C). These observations support the conclusion that hypoxia-induced changes in APD are primarily due to the SUMO-mediated increase in \(I_{LATE}\) passed by Na\(_v\),1.5 channels. Of note, baseline APD in iPSC-CMs is longer than that in native cardiomyocytes, apparently due to a lower expression of \(K^+\) channels (Yang et al., 2014).

The O’Hara-Rudy model for human cardiac action potentials was used to demonstrate that the increase in \(I_{LATE}\) due to SUMOylation of Na\(_v\),1.5 channels observed in reconstituted CHO cells and iPSC-CMs was sufficient to meet the theoretical requirements for pro-arrhythmic prolongation of APD. As anticipated, the time to repolarize the membrane from peak to rest by 50% (APD\(_{50}\)) was prolonged by 27% when \(I_{LATE}\) was increased in the model by 5-fold, half the observed maximal effect in the cells, from 205 ms under control conditions to 260 ms (Figure 5D); also as observed in iPSC-CMs, there was no appreciable change in the resting membrane potential or the action potential peak current in the model (Table S3).

**DISCUSSION**

Myocardial hypoxia increases \(I_{LATE}\) leading to increased APD, a common setting for dangerous arrhythmias. Here, we show that hypoxia induces SUMOylation of Na\(_v\),1.5 channels on K442 and this is necessary and sufficient to increase \(I_{LATE}\) and APD in human cardiomyocytes. Supporting a direct mechanism, SUMOylation of Na\(_v\),1.5 produces \(I_{LATE}\) in the absence of hypoxia in membrane patches excised from cells, basal levels of Na\(_v\),1.5 SUMOylation are low under control conditions in iPSC-CMs, and hypoxia leads to SUMOylation of the channels with the same time course as the increase in \(I_{LATE}\). Furthermore, SUMOylation produces changes in the gating of single Na\(_v\),1.5 ion channels, macroscopic \(I_{Na}\), and APDs like those described in human heart and in animal models with hypoxia (Chadda et al., 2017; Austen et al., 1963; Thung et al., 1962; Brown et al., 2014); and the effects are suppressed by the mutation of Na\(_v\),1.5 K442, the application of SENP1 deSUMOylase, or the application of ranolazine, an inhibitor of \(I_{LATE}\).

Increased \(I_{LATE}\) and AP prolongation predispose to arrhythmia because they increase the influx of Na\(^+\), raising the intracellular Na\(^+\) concentration and depolarizing the membrane potential. These changes impact the activity of other ion channels and transporters, including the Na/K-ATPase, leading to Ca\(^{2+}\) loading by L-type Ca\(^{2+}\) channel reactivation and reverse operation of the sodium-calcium exchanger (NCX). These excitatory effects increase the likelihood of early after-depolarizations (EADs), delayed after-depolarizations (DADs), triggered activity, and
Figure 5. Hypoxia-Induced Increase in APD Mediated by SUMO Modulation of \( I_{\text{LATE}} \)

Spontaneous action potentials from human iPS-CMs were recorded in current-clamp mode with the same sequence of perfusates: normoxic (21% O\(_2\), solid line), hypoxic (1.5% O\(_2\), dashed line), and then hypoxia with 1 \( \mu \)M ranolazine (purple). Action potentials were studied with control intracellular solution (black) or with 1 nM SUMO1 (red) or SENP1 (blue) in the recording pipette. APD\(_{50}\) was determined by calculating the time required for the membrane potential to return to 50% of the resting value from the peak deflection of the action potential. Hypoxia was induced as described in the STAR Methods. Data are means ± SEM for 6–9 cells studied per group (*p < 0.01, two-way paired t test). The O’Hara-Rudy model for human action potentials was applied as described in the STAR Methods and Table S3. Scale bars represent 25 mV and 250 ms in (A)–(C) and 30 mV and 150 ms in panel (D).

(A) Example spontaneous action potentials recorded from iPS-CMs studied with control pipette solution under normoxic conditions and hypoxia with and without ranolazine. The APD\(_{50}\) increased with hypoxia by 22%, from 663 ± 2 ms to 810 ± 2 ms, and application of ranolazine restored the APD\(_{50}\) to 689 ± 2 ms. (B) When SUMO1 was included in the pipette solution, APD\(_{50}\) was 622 ± 2 ms, and this increased to 870 ± 2 ms with hypoxia; ranolazine produced an APD\(_{50}\) of 699 ± 3 ms like that observed without SUMO1 in the pipette under normoxic conditions.

(C) APD\(_{50}\) was 663 ± 2 ms when cells were studied with SENP1 in the recording pipette, and the APD\(_{50}\) increased only to 698 ± 2 ms with hypoxia and was returned to 663 ± 4 ms with ranolazine.

(D) When a 5-fold increase in \( I_{\text{LATE}} \) (half the maximal effect observed with hypoxia/SUMOylation), was simulated using the O’Hara-Rudy model, the APD\(_{50}\) increased by 27%, from 205 ms in normoxia to 260 ms.

(E) Histograms summarizing the mean APD\(_{50}\) of iPS-CMs under the conditions described in (A)–(C).
open and inactive states are lowered, we observe hypoxia to increase the window between activation and steady-state inactivation voltage curves (Figure S3). Furthermore, our failure to observe long first openings suggests that the barrier to inactivation is not increased by hypoxia. Of note, destabilization of the inactivated state can rationalize \( I_{\text{LATE}} \) in association with channels that recover from inactivation too rapidly during repolarization (Belardinelli et al., 2006, 2015; Shryock et al., 2013). It is feasible that hypoxia-induced SUMOylation of Na\(_V\)1.5 on K442, a site on the linker between domains I and II, may interfere with the stability of the interaction of the IFM lid and its pore receptor.

Here, we apply SUMO1\(_{101}\) to the inner surface of excised membrane patches to SUMOylate Na\(_V\)1.5 channels, as we observed before for K2P1, K\(_V\)2.1, and Na\(_V\)1.2 channels; this indicates that enzymes stably associated with the cytoplasmic face of the plasma membrane mature SUMO1 to SUMO\(_97\) and mediate coupling to target lysine (Xiong et al., 2017; Plant et al., 2010, 2011, 2012, 2016). In contrast, when SUMOylation of Na\(_V\)1.5 channels is induced by hypoxia, the membrane must be in continuity with the cytosol, indicating cellular components have a role in transducing the signal that oxygen tension is low to the SUMO pathway. Thus far, exogenous SENP1 must be applied to deSUMOylate the ion channels in off-cell mode in all cases we have studied.

It is notable that changes in Ca\(^{2+}\) homeostasis are not required for hypoxia-induced SUMOylation of Na\(_V\)1.5 in whole cells as the reaction proceeds despite 10 mM EGTA in the recording pipette to suppress changes in cellular Ca\(^{2+}\) and 200 \( \mu\)M CdCl\(_2\) in the extracellular bath solution to suppress conductance through voltage-gated calcium channels (Figure 1A). The similar attributes of SUMOylated Na\(_V\)1.5 currents in intact cells and single channels in excised patches also support the conclusion that changes in Ca\(^{2+}\) at the cytoplasmic face of the channel are not necessary for SUMO-induced increases in \( I_{\text{LATE}} \) (Figure S5). Those points do not rule out a role for variation in cytoplasmic Ca\(^{2+}\) when the ionic concentration is not clamped by chelation and blockade.

Ion channel SUMOylation appears to enhance excitability by decreasing potassium currents (K2P1, K\(_V\)2.1, and Kv7.1/KCNNE1) (Rajan et al., 2005; Plant et al., 2010, 2012) or increasing currents passed by Na\(_V\)1.2 (Plant et al., 2016) and Na\(_V\)1.5 (this work). Our prior study showing that acute hypoxia leads to the rapid SUMOylation of Na\(_V\)1.2 in CGN and influx of Na\(^+\) flux due to altered voltage-dependent gating (but not a change in \( I_{\text{LATE}} \)) (Plant et al., 2016) and this study of Na\(_V\)1.5 suggest that hypoxia-mediated SUMOylation is an emerging common mechanism shared by some, but not all, ion channels.

We observed previously that acidosis increases \( I_{\text{LATE}} \) in Na\(_V\)1.5 channels bearing a common polymorphism associated with SIDS and arrhythmia in African American adults, presenting a model for the genetic predisposition to dysrhythmia in the face of an environmental challenge (Plant et al., 2006). Inherited ion channel mutations that predispose to arrhythmia by increasing APD also yield life-threatening events in response to specific stimuli, for example, concurrent drug blockade (Sesti and Goldstein, 1998). Here, we present the mechanism for the sensitivity of wild-type Na\(_V\)1.5 channels to the environmental challenge of hypoxia. Identifying the role of the SUMO pathway in the increase in \( I_{\text{LATE}} \) in response to hypoxia supports targeting the pathway for therapeutic intervention.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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  - DATA AND CODE AVAILABILITY

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.01.025.

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**AUTHOR CONTRIBUTIONS**

L.D.P., D.X., J.R., and H.D. performed the research and analyzed data; L.D.P. and S.A.N.G. designed the research and wrote the paper.

**DECLARATION OF INTERESTS**

The authors report no competing financial interests.

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STAR★METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and Virus Strains** |        |            |
| BL21(DE3) E. Coli | Thermo Fisher | Cat#: EC0114 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Human: SUMO | Boston Biochem | Cat #: UL-712 |
| Human: His6-SENP1 catalytic domain | Boston Biochem | Cat#: E-700 |
| Ranolazine dihydrochloride | Sigma-Aldrich | Cat#: R6152 |
| **Experimental Models: Cell Lines** |        |            |
| Hamster: CHO-K1 cells | ATCC | Cat#: CCL-61; RRID: CVCL_0214 |
| Human: iPS-ventricular cardiomyocytes-2 | Cellular Dynamics | Cat# CMC-100-012-000.5 |
| **Recombinant DNA** |        |            |
| hNa1.5-pcDNA1 | Plant et al., 2006 | DOI: 10.1172/JCI25618 |
| GFP-SCN1B-pcDNA1 | Plant et al., 2006 | DOI: 10.1172/JCI25618 |
| mCherry-SUMO1-pMAX | Plant et al., 2016 | https://doi.org/10.7554/eLife.20054 |
| mTFP1-hNav1.5-pMAX | This paper | N/A |
| eYFP-SUMO1-pMAX | Plant et al., 2010 | https://doi.org/10.1073/pnas.1004712107 |
| eYFP-Ubc9-pMAX | Plant et al., 2010 | https://doi.org/10.1073/pnas.1004712107 |
| Plasmid for MS with SUMO1(T95K)1-97 | Plant et al., 2010 | https://doi.org/10.1073/pnas.1004712107 |
| Plasmid for MS with Human NaV1.5 residues 353-502 | Plant et al., 2010, modified as described | https://doi.org/10.1073/pnas.1004712107 |
| **Software and Algorithms** |        |            |
| pClamp version 10 | Molecular Devices | N/A |
| Origin version 6 | Microcal | N/A |
| Prism version 8 | GraphPad | N/A |
| ImageJ-FIJI | Schindelin et al., 2012; https://imagej.net/ Fiji | https://doi.org/10.1038/nmeth.2019 |
| MSConvert | http://proteowizard.sourceforge.net/tools.shtml |        |
| Batch-Tag in Protein Prospector v. 6.1.0 | http://prospector.ucsf.edu/ |        |
| O’Hara Rudy Model (ORD) | O’Hara et al., 2011 | https://doi.org/10.1371/journal.pcbi.1002061 |

LEAD CONTACT AND MATERIALS AVAILABILITY

Further Information and requests for resources and reagents should be directed to the Lead Contact, S.A.N. Goldstein (sgoldst2@uci.edu). Materials generated through this work are available from the Lead Contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

CHO-K1 cells (RRID: CVCL_0214) were obtained from ATCC and used for heterologous expression, as detailed below. Human induced pluripotent stem cell cardiomyocytes were purchased from Cellular Dynamics and cultured on #1.5 glass coverslips coated with gelatin (0.1% w/v in water) according to the manufacturer’s instructions. Cells were maintained in iCell maintenance medium (Cellular Dynamics) and incubated at 37°C in a humidified atmosphere containing 5% CO₂ / 95% air (21% O₂). The iPSC cells were studied between days 5 - 10 in culture at which time both isolated cells and cells in syncytia exhibited rhythmic beating behavior, a criterium for mature myocyte attributes shown to correspond with expression of troponin-T and sarcomeric α-actinin (Ma et al., 2011). To further verify that the currents under study in iPSC cells were mediated by NaV1.5 channels, we employed protocols and
solutions to minimize conductance through potassium and calcium channels (per Figures 1, S1, and S3); showed that the currents had biophysical hallmarks like those of human Na₉.1.5 in tissue culture cells (per Table 1 and Figures 3 and S3); and demonstrated blockade with ranolazine, a drug that inhibits Iᵢ₄mediate by Na₉.1.5 (per Table S3 and Figure S3).

METHOD DETAILS

Molecular Biology and Reagents

Human Na₉.1.5 (NM_198056.1) was handled in pcDNA1, as previously described (Plant et al., 2006) and was transiently cotransfected with GFP-tagged SCN1B (the β subunit of cardiac SCN5A channel, isoform b; GenBank: NM_001037). Human SUMO1₁₅ (GenBank: NM_003352.8) and Ubc9 (GenBank: NM_003345.5) were amplified from a brain cDNA library (Clontech) and inserted into pMAX, as described before (Rajan et al., 2005). Sequences encoding mCherry or mTFP1 were inserted as described (Plant et al., 2010) at the N terminus of SUMO1 or Na₉.1.5 respectively. SUMO1 and Ubc9 were tagged with eYFP using the same strategy. Mutations were introduced with Pfu Quikchange PCR (Agilent). Purified SUMO1₁₇, SUMO2₉₃, SENP1 and SENP2 were purchased from Boston Biochemical.

Heterologous Expression

CHO-K1 cells (RRID: CVCL_0214) were maintained in F12K medium supplemented with 10% FBS. Plasmids were transfected into cells with Lipofectamine 2000 according to the manufacturer’s instructions (Life Technologies). Experiments were performed 24 to 48 hours post transfection at room temperature. The human β1 accessory subunit was co-expressed with Na₉.1.5 subunits unless otherwise indicated.

Electrophysiology

Iᵢ₄ and action potentials in iPS-CMs, and whole-cell Na₉.1.5 channel currents in heterologous cells were recorded using an Axopatch 200B amplifier and pCLAMP software (Molecular Devices) at filter and sampling frequencies of 10 and 50 kHz respectively. For voltage-clamp studies, cells were superfused with a solution comprising, in mM: NaCl 130, CsCl 4, CaCl₂ 2, MgCl₂ 1.2, glucose 5.5, HEPES 10 and, 200 μM CdCl₂, a concentration previously shown to block >98% of voltage-gated calcium channel current in primary cells (Pearson et al., 1993). The pH was adjusted to 7.4 with NaOH and HCl. Cells were studied at room temperature with borosilicate glass pipettes (Clark Kent) with a resistance of 2-3 MΩ when filled with a solution comprising, in mM: CsCl 60, CsF 80, CaCl₂ 1, MgCl₂ 1, Na₂ATP 5, EGTA 10, HEPES 10, pH 7.4 with CsOH. Pipettes were coated with Sylgard (Dow Corning) prior to use. Capacitance artifacts were subtracted online, series resistance was compensated to 70% and cells with a series resistance of less than 10 MΩ were studied. Once whole-cell mode was established, cells were not studied for more than 600 s in order to maintain consistent membrane seals and voltage-clamp control of Iᵢ₄. Current-voltage relationships were evoked from a holding potential of −100 mV by 100 ms test pulses between −100 and 0 mV, in 10 mV increments. Steady-state inactivation was studied by holding cells at −140 mV and then comparing currents evoked by 50 ms test pulses between −140 mV and −20 mV to those evoked by a 100 ms prepulse to 0 ms. A 10 s interpulse interval was used in both cases. Normalized peak current values are plotted against prepulse potential (mV). A Boltzmann function, $I = I_{\text{max}} / (1 + \exp[V - V_{1/2} / k])$, where $I_{\text{max}}$ is the maximum current and $k$ is slope factor, was used to fit normalized activation-voltage relationships. Recovery from fast inactivation was studied by holding cells at −100 mV and comparing currents evoked by a pair of 50 ms test step to −30 mV separated by an interpulse interval that increased in duration by 5 ms increments per sweep. The time constant for recovery from fast inactivation (τ) was obtained from mono-exponential fits of the normalized current amplitude to the recovery time using $I = I_{\text{max}} + A e^{-t/\tau}$, where $A$ is the amplitude of components and $\tau$ is time. Whole-cell currents were normalized to cell capacitance. Mean ± SEM capacitance values were 28 ± 5 pF for iPS cells and ± 2 pF for CHO cells.

For current-clamp studies, iPS-CMs were superfused with a solution comprising, in mM: NaCl 135, KCl 3.5, CaCl₂ 2, MgCl₂ 1.2, glucose 8, HEPES 10 and the pH was adjusted to 7.4. The intracellular solution contained, in mM: KCl 140, MgCl₂ 2, Na₂ATP 5, EGTA 1, HEPES 1 and the pH was adjusted to 7.4.

Acute Hypoxia for Electrophysiology

Acute hypoxia was achieved as described previously (Plant et al., 2002). Briefly, the cells were made hypoxic (1.5% O₂) by switching the perfusate with one that had been bubbled with nitrogen for at least 30 minutes prior to perfusion. Oxygen tension was measured at the cell by a polarized carbon fiber electrode; solution exchange occurred in less than 10 s. Ambient O₂, or normoxia recording conditions, were achieved by allowing recording solutions to equilibrate with room air for at least 30 minutes prior to perfusion. The ambient O₂ solution was measured to be 21% O₂ at the cell. All solutions were equilibrated, measured, and perfused at room temperature (18°C).
a 520-nm dichroic mirror mounted in a DualView adaptor (Photometrics) and each wavelength was directed to one half of an EM-CCD. The dichroic mirror was disengaged when single fluorophores were studied.

To assess stoichiometry, fluorophores were photo-bleached by continual excitation and data were captured as movies of 100–400 frames acquired at 1 Hz using an EM-CCD camera (Hamamatsu). When mTFP1 was studied with mCherry in the same cell, the data for each fluorophore were saved as separate stacks and processed in an identical manner. Images were background corrected by subtracting the mean of 5 fully bleached frames from the end of each stack analyzed. Misalignment of the data between stacks was corrected in ImageJ using StackReg. Fluorescent spots were defined as a discrete 3 × 3-pixel region around a pixel of maximum intensity, as before (Plant et al., 2010, 2014). Fluorescence is reported as the change in fluorescence intensity normalized by the initial fluorescence for each trace. The density of co-localized and single fluor-fluorescent particles was determined following thresholding and watershed separation in ImageJ. The particle number was counted in 4, separate 100 × 100-pixel regions of interest for 6–10 cells per group using the Analyze particles plugin.

Manders’ coefficient of colocalization was assessed from live-cell simultaneous two-color TIRF images captured at 5 s intervals to minimize photobleaching. Data stacks were background subtracted and aligned prepared for each fluorophore post hoc, as above. Co-localization of partner pixels from the two stacks of images was defined as the presence of both fluorophores with at least 30% of maximum fluorescence levels recorded in that region of interest. Mean Manders’ coefficients were calculated for 3–5 separate 100 × 100-pixel regions of interest per cell.

**Live Cell FRET Microscopy**

Donor-decay time-course was studied as before (Plant et al., 2010), using an Olympus inverted epi-fluorescence microscope. Cells were studied in a solution comprising (in mM): NaCl 130, KCl 4, MgCl₂ 1.2, CaCl₂ 2, HEPES 10, pH was adjusted to 7.4 with NaOH. mTFP1 was excited at 445 nm and the emission collected through a 470–500 nm bandpass filter, YFP was excited at 514 nm and the emission collected through a 525–575 nm filter. Images were captured using a scientific camera controlled by µmanager or Metamorph software (Molecular Devices) and were analyzed with ImageJ.

**Mass Spectrometry**

Human Na₉,1.5 residues 353-502 were cloned into pET28a vector with six-His residues and a tobacco etch virus (TEV) cleavage site replacing the thrombin site and expressed in BL21(DE3) E. Coli with a vector carrying mouse E1 (as a linear fusion product of Aos1 and Uba2), E2 (Ubc-9) and SUMO1/2 with a T95K mutation so that trypsin digestion before MS leaves a Gly-Gly tag on the ε-amino group of target lysines as before (Plant et al., 2010). Protein was purified with Ni-NTA affinity columns. The eluate was reduced with TCEP and alkylated using iodoaceticamide prior to tryptic digestion via filter-assisted digestion. LC MS/MS analysis was performed using a Thermo Scientific Dionex UltiMate 3000 system coupled in-line to an Orbitrap Fusion Lumos MS. A 25 cm x 75 µm PepMap EASY-Spray Acclaim PepMap C18 Column was used to separate peptides over acetonitrile gradients of 4% to 28% at a flow rate of 300 nL/min. Each duty cycle consists of one MS scan in FT mode (350-1500 m/z, resolution of 120,000 at m/z 400) followed by data-dependent tandem mass scans in IT mode for 3 s at top speed, utilizing HCD with NCE 30% on the most intense ions with charge states ≥2 or higher. The RAW data file acquired was extracted to MGF format using MSConvert (http://proteowizard.sourceforge.net/tools.shtml) and subjected to database searching using Batch-Tag within a developmental version of Protein Prospector (v. 6.1.0) against a decoy database consisting of a normal Homo sapiens Swissprot database concatenated with a randomized version (SwissProt.2019.4.8.random.concatenate, total 20,418 protein entries). The mass accuracy for parent ions and fragment ions were set as ± 10 ppm and 0.2 Da, respectively. Trypsin was set as the enzyme and the maximum of two missed cleavages were allowed. Protein N-terminal acetylation, methionine oxidation, Gly-Gly, and N-terminal conversion of glutamine to pyroglutamic acid were selected as variable modifications. Minimum protein and peptide scores were set as 22.0 and 5.0 respectively, while maximum E values for proteins and peptides were set to 0.01 and 0.1. A list of identified precursor ions corresponding to peptides carrying GlyGly modifications were generated for targeted sequencing for further validation. All MS/MS spectra for potential SUMOylated peptides were further inspected manually and evaluated based on spectral quality.

**Simulations**

Action potentials were simulated using the O’Hara Rudy (ORD) model (O’Hara et al., 2011) under normal condition or by scaling the conductance of Iₙa,late. To simulate the effect of SUMO (or hypoxia-induced SUMOylation) a scaling factor named “SUMO” that varies from 1 (No SUMO) to 10 (maximum effect at 1 nM SUMO1) was added to the model to increase the conductance of Iₙa,late. SUMO = Gₙa,late * SUMO, were Gₙa,late is the nominal conductance value from the O’Hara-Rudy ventricular myocyte model and Gₙa,late SUMO is the conductance used for simulation in the modified ORD. Other variables and all equations of the model were otherwise as described in the published model, except that the external ionic concentrations were set to match the experimental conditions used here to study the iPS-CMs (in mM): [Na⁺]₀ = 135; [Ca²⁺]₀ = 2.0; and [K⁺]₀ = 3.5. A pacing cycle length of 800 ms was used and the numbers of beats was set to 1000. The duration of the APD₅₀ was measured from the time of maximum dV/dt to the time that membrane voltage reached 50% of complete repolarization; resting potential was measured immediately before each beat.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data were analyzed using pClamp, Origin, GraphPad and Excel software. Quantification and analysis specific in each experimental technique is described in the Method Details section, above. Data were assessed for statistical differences between groups by
one-way analysis of variance with Bonferroni post hoc analysis to test differences within pairs of group means for all dataset with an F-value of $p < 0.05$. Data are presented, where indicated as the mean ± standard error of the mean (SEM). The number of replicates for each study are described in the legends.

DATA AND CODE AVAILABILITY

The study did not generate any unique datasets or codes.