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SHORT REPORT

Oxygen-dependent hydroxylation by FIH regulates the TRPV3 ion channel

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ABSTRACT

Factor inhibiting HIF (FIH, also known as HIF1AN) is an oxygen-dependent asparaginyl hydroxylase that regulates the hypoxia-inducible factors (HIFs). Several proteins containing ankyrin repeat domains (ARDs) have been characterised as substrates of FIH, although there is little evidence for a functional consequence of hydroxylation on these substrates. This study demonstrates that the transient receptor potential vanilloid 3 (TRPV3) channel is hydroxylated by FIH on asparagine 242 within the cytoplasmic ARD. Hypoxia, FIH inhibitors and mutation of asparagine 242 all potentiated TRPV3-mediated current, without altering TRPV3 protein levels, indicating that oxygen-dependent hydroxylation inhibits TRPV3 activity. This novel mechanism of channel regulation by oxygen-dependent asparaginyl hydroxylation is likely to extend to other ion channels.

KEY WORDS: FIH, TRPV3, Hydroxylation, Hypoxia

INTRODUCTION

Oxygen sensing is fundamental to all metazoa, and contributes to development, normal physiology and disease. In mammals, many acute responses to hypoxia are mediated by ion channels, whereas chronic responses to hypoxia commonly result in altered gene expression, mediated by transcription factors such as hypoxia inducible factors (HIFs).

Factor inhibiting HIF (FIH, also known as HIF1AN) is a 2-oxoglutarate-dependent dioxygenase that regulates the activity of the HIFs through oxygen-dependent hydroxylation (Hewitson et al., 2002; Lando et al., 2002a; Lando et al., 2002b; Mahon et al., 2001). Several proteins containing ankyrin repeat domains (ARDs) are substrates for asparaginyl hydroxylation by FIH, including IKβα, p105, Notch and ASPP2 (also known as TP53BP2) (Cockman et al., 2006; Cockman et al., 2009; Coleman et al., 2007; Janke et al., 2013; Zheng et al., 2008), but the functional consequences of hydroxylation remain unclear. Despite this, FIH-deficient mice display elevated metabolism, insulin hypersensitivity, hyperventilation and reduced body weight (Zhang et al., 2010), but no disruption to classical HIF-regulated pathways such as those regulating angiogenesis, consistent with a role for non-HIF targets of FIH.

We performed a bioinformatic search to identify novel substrates of FIH, and identified members of the transient receptor potential (TRP) ion channel family. We demonstrate that the TRP vanilloid-3 channel (TRPV3) is hydroxylated by FIH, leading to oxygen-dependent regulation of TRPV3-mediated current.

RESULTS AND DISCUSSION

A bioinformatic search for novel FIH substrates was performed using a consensus sequence generated from known FIH substrates (Wilkins et al., 2012). TRP channels, non-selective cation channels permeable to Ca^{2+} that perform a range of sensory roles (Ramsey et al., 2006; Venkatachalam and Montell, 2007), were identified as likely substrates with predicted hydroxylation sites within the cytoplasmic ARDs (Fig. 1A). The best candidate was TRPV3, a channel that is most highly expressed in the skin, central nervous system, testis and gastrointestinal tract, with roles in thermosensation, nociception, skin barrier formation, hair morphogenesis and wound healing (Nilius et al., 2014).

To investigate the ability of FIH to hydroxylate TRPV3 *in vitro*, recombinant mouse and human TRPV3-AR3–6 were analysed for hydroxylation using the [^{14}C]-2-oxoglutarate capture assay (Linke et al., 2007). The TRPV3-ARD polypeptides produced activity with FIH that was similar to that of the well-characterised Notch1-ARD substrate (Coleman et al., 2007; Zheng et al., 2008), providing strong evidence that TRPV3 is a substrate of FIH (Fig. 1B). TRPV4 displayed activity slightly above that of the Notch1-N1945A/N2012A negative control (Zheng et al., 2008), consistent with FIH inefficiently hydroxylating histidine residues in ARD substrates (Yang et al., 2011).

Mutation of the predicted target asparaginyl residue to alanine in mouse (m)TRPV3 (N242A) reduced activity to background levels (Fig. 1C), consistent with N242 being the predominant site of hydroxylation. Affinity pull-down assays (Fig. 1D) demonstrated that recombinant ARDs from TRPV3, but not TRPV4, efficiently bound to FIH. Importantly, FIH-mediated hydroxylation of TRPV3 was oxygen-dependent, with activity greatly reduced in 0.5% oxygen (Fig. 1E). The retention of some activity in severe hypoxia is not surprising, given that FIH can remain active under conditions of severe hypoxia (0.2% oxygen) (Singleton et al., 2011; Tian et al., 2011).

To confirm hydroxylation *in vitro*, recombinant TRPV3-ARD expressed in *Escherichia coli* was analysed by mass spectrometry (MS) before and after incubation with recombinant FIH. An ion

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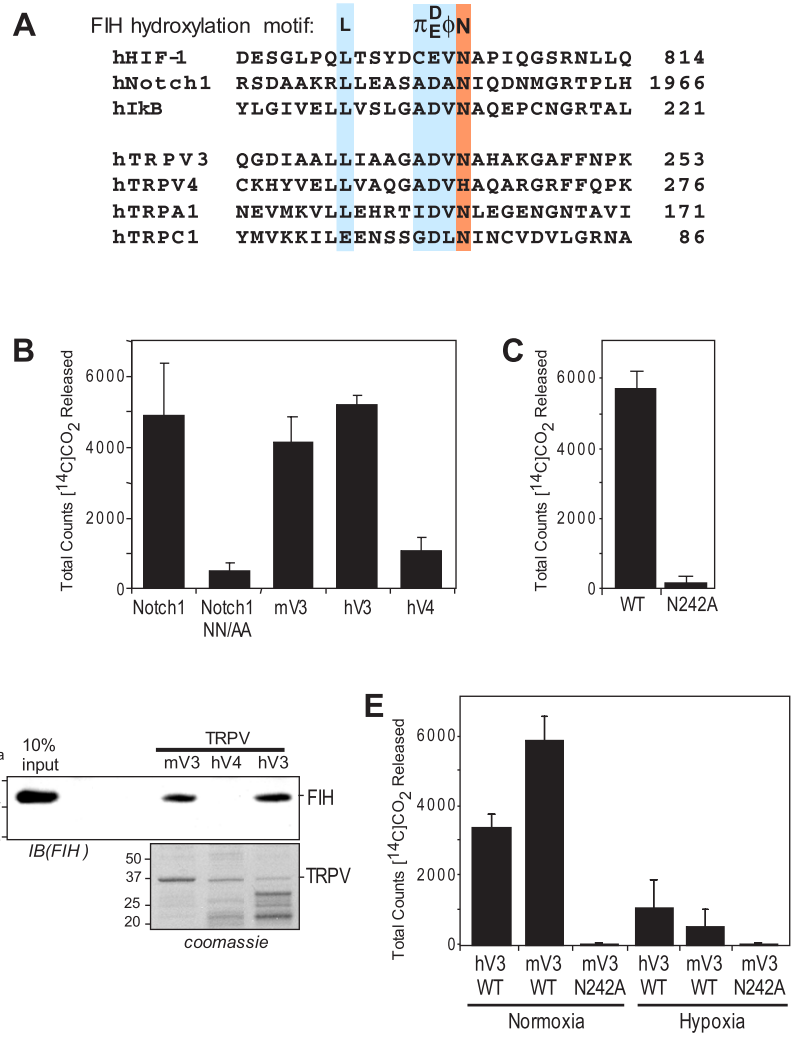

Fig. 1. Oxygen-dependent hydroxylation of TRPV3 by FIH *in vitro*. (A) Results from a bioinformatic search of the National Center for Biotechnology Information (NCBI) database for the LXXXXπD/EφN consensus motif (blue and orange highlighting) using the blastp algorithm. π represents a small, uncharged residue and φ represents a hydrophobic residue. (B,C) CO_2 capture assay with purified MBP–FIH and Trx-6his–TRPV-ARD proteins with positive (mNotch1) and negative [Notch1-NN/AA (mNotch1-N1945A/N2012A)] controls and Trx-6his–TRPV-ARD-N242A (N242A). mV3, mouse TRPV3; hV3, human TRPV3; hV4, human TRPV4; WT, wild type. (D) Affinity pull-down assay with purified Trx-6his–TRPV-ARD proteins attached to Ni-NTA resin used to pull-down endogenous mFIH from mouse embryonic fibroblast whole-cell extracts. IB, immunoblot. Data are representative of two independent experiments. (E) CO_2 capture assay as in B,C under conditions of normoxia (21% O_2) and hypoxia (0.5% O_2). CO_2 capture assays were performed in triplicate, and are displayed as the mean±s.d. (at least three independent experiments).

corresponding to the unmodified tryptic peptide containing N242 was detected from untreated TRPV3 [mass to charge ratio (m/z) 1919.096]. Incubation of this peptide with FIH resulted in a modified TRPV3 peptide ion (m/z 1935.079). MALDI-TOF/TOF-MS/MS analyses (Fig. 2A–C) indicated that N242 was modified by the addition of one oxygen atom, as unmodified y5–17 fragment ions could be assigned to the unmodified peptide, but those from the modified peptide contained an additional oxygen atom.

Hydroxylation within cells was investigated by transfecting HEK293T cells with full-length TRPV3 fused to an N-terminal Myc 6-histidine (6his) tag together with FIH. These cells were then treated with or without dimethyloxalyl glycine (DMOG), an inhibitor of FIH (Lando et al., 2002a), similar to the functional experiments shown in Fig. 4. Myc-6his–TRPV3 was affinity purified, and high-performance electrospray ionisation mass spectrometry (ESI-MS) of tryptically digested TRPV3 detected doubly charged ions corresponding to both hydroxylated and nonhydroxylated peptides containing N242. Tandem mass spectrometry of these doubly charged peptide ions revealed ion series (Fig. 2D,E) similar to the y ion fragment series from the hydroxylated and nonhydroxylated recombinant proteins described above (Fig. 2B,C), thus confirming hydroxylation of full-length TRPV3 at N242 within cells. As expected, DMOG greatly reduced hydroxylation, with the ratio of hydroxylated to nonhydroxylated TRPV3 peptides decreasing ~30-fold from 1.2 to 0.04 after treatment (Fig. 2F), consistent with hydroxylation being FIH-dependent. Hydroxylation of N242 was also detected in HEK293T cells stably expressing TRPV3 (data not shown).

To investigate the function of TRPV3 hydroxylation by FIH, HEK293T cells were co-transfected with full-length TRPV3 and GFP. Whole-cell patch recordings showed robust activation of TRPV3-mediated current in response to application of the TRPV3 agonist, 2-aminoethoxydiphenyl borate (2-APB) (Fig. 3A,B) (Chung et al., 2004; Hu et al., 2004). With each consequent application of 2-APB, the current amplitude increased, saturating after four to five applications (Fig. 3A). The current–voltage plot showed characteristic outward rectification (Fig. 3B) (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002). Current with the same properties was recorded in HEK293T cells stably transfected with Myc-6his–TRPV3 used in the mass spectrometry analysis.

Of considerable interest is whether TRPV3 channels are regulated by hypoxia, with a role for oxygen-dependent hydroxylation mediated by FIH. To address this question, HEK293T cells were transfected with TRPV3 and preincubated under hypoxic conditions (0.1% oxygen) for 24 h prior to patch clamping, to inhibit endogenous FIH and thus prevent hydroxylation of TRPV3. Severe hypoxia was used to ensure inhibition of FIH (Singleton et al., 2011). Hypoxia substantially increased the TRPV3 current amplitude above that observed during normoxia (Fig. 3C); the increase in amplitude was sustained over

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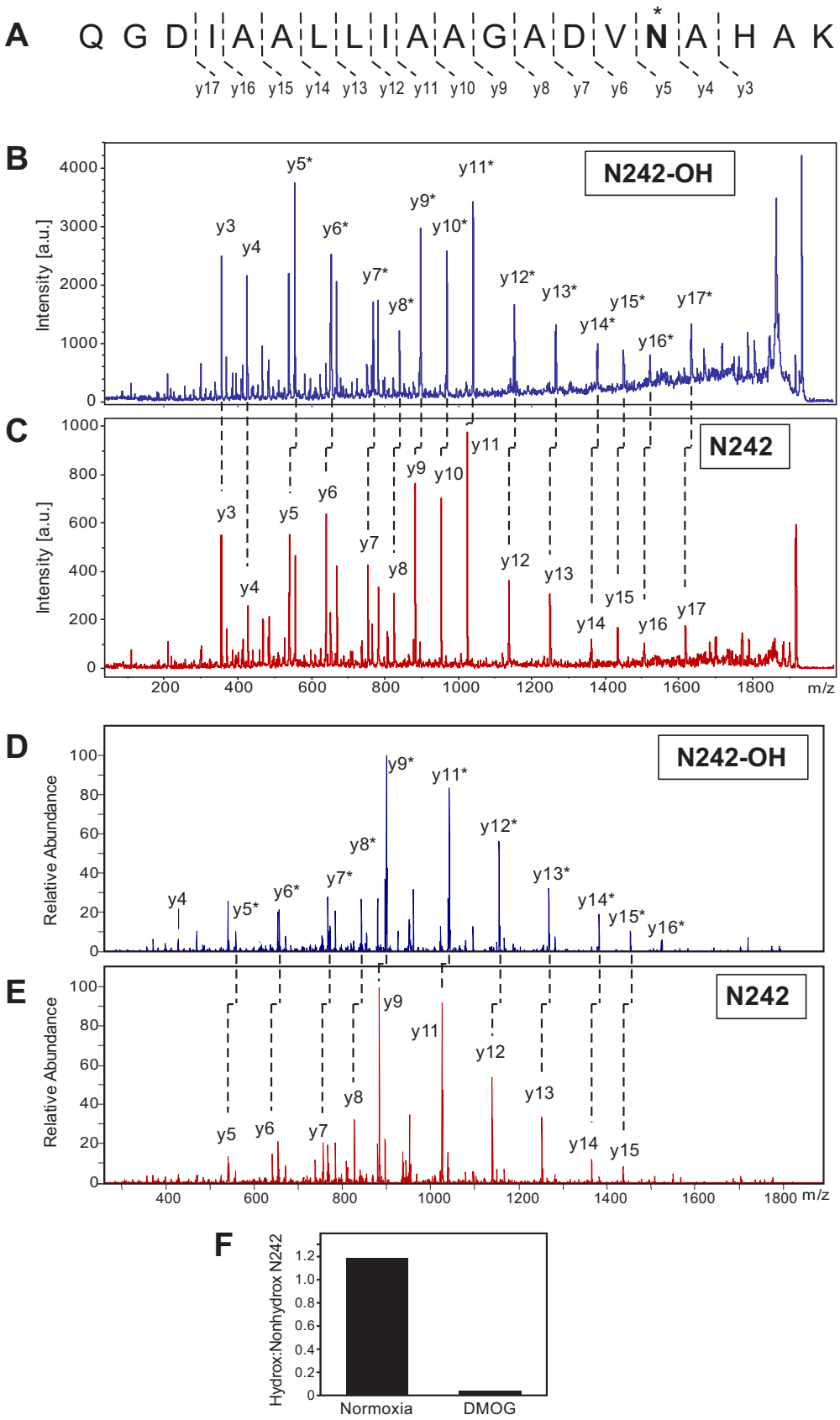

Fig. 2. Demonstration of hydroxylation of N242 of TRPV3 by tandem mass spectrometry. (A) The tryptic peptide corresponding to residues 227–246 of TRPV3, with hydroxylated asparagine highlighted with an asterisk. (B,C) Tandem MALDI-TOF/TOF-MS/MS spectra were obtained from the hydroxylated (B) and non-hydroxylated (C) peptides. Isobaric correspondence between the y3 and y4 fragment ions in both spectra and subsequent shifts of 16 atomic mass units (amu) for all subsequently identified y fragment ions of the hydroxylated precursor ion relative to the non-hydroxylated precursor ion are indicated by dashed lines. The fragment ions containing hydroxylated asparagine are indicated by asterisks. a.u., arbitrary units. (D,E) Tandem mass spectrometric spectra, similar to those shown in B,C were obtained by nanoHPLC-LTQ-Orbitrap analysis of a tryptic peptide (m/z=968.0176) from Myc-6his–TRPV3 isolated after expression under normoxia in HEK293T cells. (F) Quantification of hydroxylated and nonhydroxylated tryptic peptides (A) in HEK293T cells co-transfected with Myc-6his–TRPV3 and FIH, with and without 1 mM DMOG treatment for 16 h.

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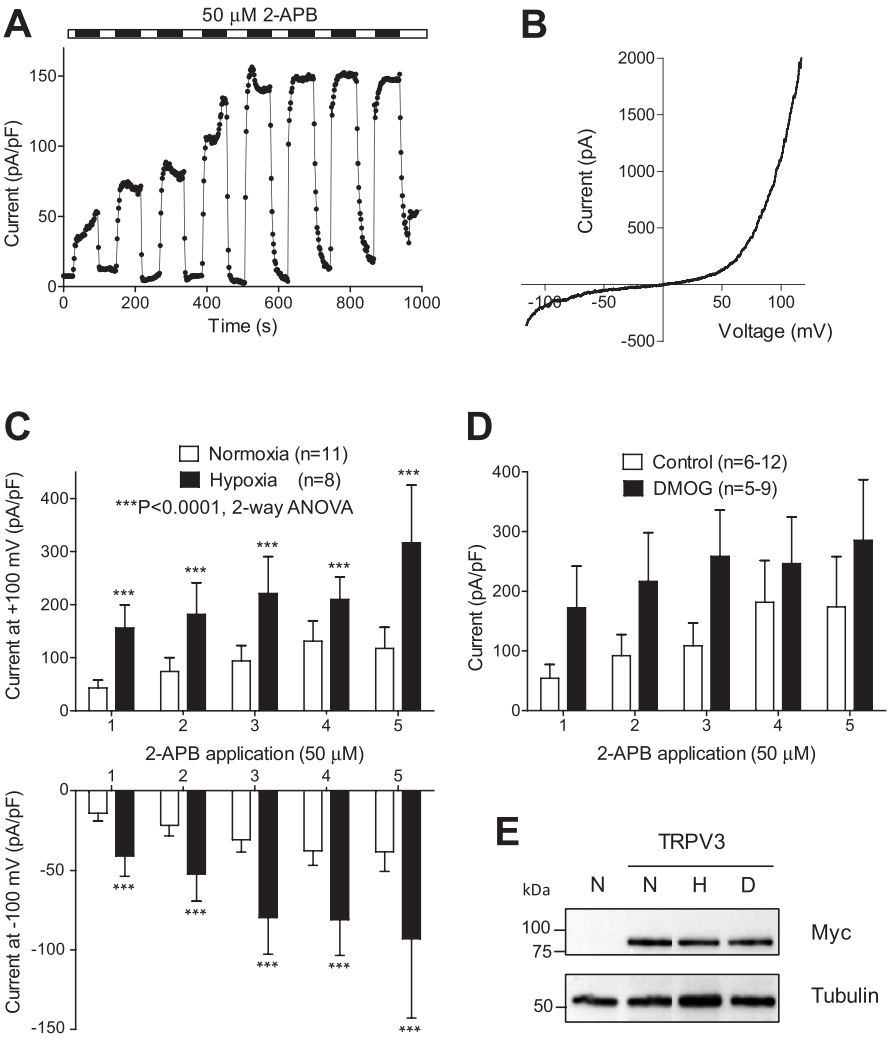

Fig. 3. Heterologously expressed TRPV3 channels are regulated by hypoxia.
(A) Timecourse of outward TRPV3 current recorded in response to repeated applications of 2-APB (black bars). Each point represents the amplitude of current at 100 mV taken from the I–V plots recorded in response to voltage ramps between −120 and 120 mV applied every 2 s. Current was normalised to cell capacitance. (B) Example I–V plot of TRPV3 current recorded in response to a voltage ramp. (C) Average amplitude of TRPV3 outward (upper panel) and inward (lower panel) currents recorded at +100 and −100 mV, respectively, in response to five consecutive applications of 2-APB from cells incubated for 18 h under conditions of normoxia (n=11) or hypoxia (1% O_2) (n=8). The data are from HEK293T cells stably transfected with TRPV3. The effect of hypoxia was significant (P<0.0001; two-way ANOVA). (D) Average maximum amplitude of TRPV3 currents recorded in response to five consecutive applications of 2-APB from cells incubated for 18 h under control conditions (n=6–12) or with 1 mM DMOG (n=5–9). The effect of DMOG was significant (P=0.0077; two-way ANOVA). Data in C,D show the mean±s.e.m. (≥3 independent transfections). (E) Western blot of Myc–hTRPV3 expression under conditions of 16 h normoxia ('N'), hypoxia ('H', 0.1% O_2) or 1 mM DMOG ('D'). Data are representative of three independent experiments.

repetitive applications of 2-APB (Fig. 3C). Cells were also treated with DMOG with similar results; an increase in TRPV3-mediated current was observed after DMOG treatment (Fig. 3D), consistent with hydroxylation by endogenous FIH inhibiting channel activity in normoxia and mediating a response of TRPV3 channels to hypoxia. This is a change in channel activity, as total TRPV3 protein levels do not change when the cells are incubated under hypoxic conditions or treated with DMOG (Fig. 3E).

Next, FIH and TRPV3 were co-transfected into HEK293T cells to achieve similar levels of expression. Treatment with DMOG increased the TRPV3 current amplitude in the presence of overexpressed FIH (Fig. 4A), consistent with the DMOG-dependent decrease in hydroxylation demonstrated by mass spectrometry (Fig. 2F) increasing TRPV3 activity. Overexpression of the catalytically inactive mutant FIH-H199A (Lando et al., 2002a), to outcompete endogenous FIH, resulted in similar TRPV3 activity to that observed following overexpression of FIH in the presence of DMOG (Fig. 4A). These data demonstrate that FIH-mediated hydroxylation inhibits TRPV3 activity, with reduced levels of hydroxylation caused by hypoxia, DMOG treatment or the presence of inactive FIH all increasing TRPV3 current. Unlike other ARD substrates of FIH, where hydroxylation-dependent changes in substrate activity are either subtle or are not detected, this is a clear demonstration of an ARD substrate being regulated by FIH-mediated hydroxylation.

To confirm the role of TRPV3 hydroxylation at N242, HEK293T cells were transfected with the hydroxylation-deficient TRPV3-N242A mutant (Fig. 1C), and the increase in current amplitude in response to hypoxia was greatly diminished (Fig. 4B), consistent with hypoxic regulation being mediated predominantly by oxygen-dependent FIH-mediated hydroxylation at N242. Although TRPV3-N242A cannot be hydroxylated by FIH, mutation of this well conserved asparaginyl residue might also influence the ARD structure, complicating the interpretation of the normoxic activity of this mutant.

Finally, the hydroxylation-dependent response to acute hypoxia was investigated. HEK293 cells stably expressing TRPV3 or TRPV3-N242A were exposed to hypoxia [pO_2=20.2±1.7 mmHg (±s.e.m.), equivalent to ~3% oxygen, the lowest level achieved in these acute hypoxia experiments] for 5 min (Fig. 4C). TRPV3 current amplitude after application of 2-APB increased by more than twofold after 4 min of hypoxia [Fig. 4D, 63.1±13.7 pA/pF (normoxia) to 208.3±25.8 pA/pF (hypoxia), P<0.001, n=11]. In contrast, the TRPV3-N242A mutant showed only a small increase in response to hypoxia [Fig. 4E, 148.5±18.6 pA/pF (normoxia) to 213.1±34.7 pA/pF (hypoxia), P<0.05, n=12], confirming a role for FIH-dependent hydroxylation in the acute response of TRPV3 to hypoxia.

These data identify a non-HIF substrate of FIH that is regulated in an oxygen-dependent manner by hydroxylation. Although

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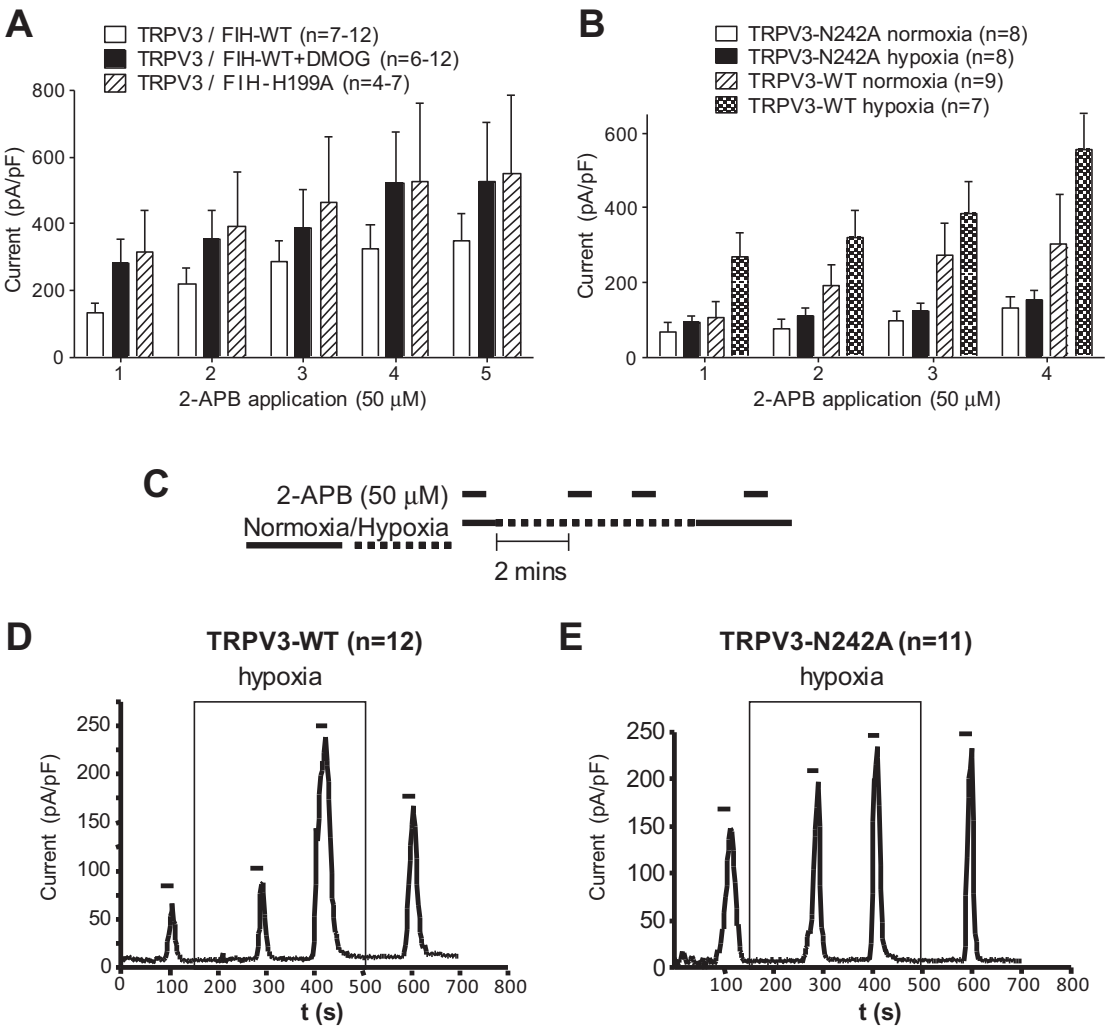

Fig. 4. Specific mutations in TRPV3 and FIH abolish hypoxic regulation of TRPV3 current. (A) Average maximum amplitude of TRPV3 currents recorded in response to five consecutive applications of 2-APB in HEK293T cells co-transfected with TRPV3 and either wild type (WT) FIH or FIH-H199A. Cells were incubated for 18 h in the presence or absence of 1 mM DMOG. Effects of DMOG and FIH-H199A mutation were significant compared to FIH-WT (P=0.0065 and 0.0071, correspondingly; two-way ANOVA). There was no difference between FIH-WT+DMOG and FIH-H199A (P=0.7; two-way ANOVA). (B) Average maximum amplitude of TRPV3 currents in response to four consecutive applications of 2-APB in HEK293T cells transiently expressing TRPV3-WT or TRPV3-N424A, incubated for 18 h in normoxia or hypoxia (1% O_2). The effect of hypoxia was significant for TRPV3-WT (P=0.0042; two-way ANOVA) but not for the TRPV3-N424A mutant (P=0.14; two-way ANOVA). Data in A,B show the mean±s.e.m. (≥3 independent transfections). (C) Schematic of the bath perfusion protocol during whole-cell patch clamping undertaken to determine the effect of acute hypoxia on TRPV3 current. (D,E) Example timecourse plots of TRPV3 current, measured at +100 mV, evoked by application of 2-APB (50 μM, black bars) under normoxia and hypoxia (pO_2 20.2±1.7 mmHg) for hTRPV3-WT (left) and TRPV3-N242A (right).

previous studies have identified numerous ARD proteins as substrates of FIH (Cockman et al., 2006; Cockman et al., 2009; Coleman et al., 2007; Singleton et al., 2011; Zheng et al., 2008), hydroxylation has no clear effect on their activity. This led to the hypothesis that the ARD substrates act to sequester FIH and consequently modulate HIF hydroxylation and activity (Coleman et al., 2007). In contrast, hydroxylation of the TRPV3-ARD clearly influences the TRPV3 channel directly. These data, together with a recent report on ASPP2 (Janke et al., 2013), demonstrate that FIH can directly influence the activity of specific ARD substrates by hydroxylation.

Importantly, given the well-documented role of ion channels in mediating physiological responses to hypoxia, these findings demonstrate that hydroxylation by FIH can mediate both acute and chronic responses to hypoxia, in common with the HIF prolyl hydroxylases (PHDs), which regulate TRPA1 channel activity (Takahashi et al., 2011).

Hydroxylation could alter channel multimerisation, interactions with other proteins that can modulate channel gating or localisation in the plasma membrane. The recent determination of the TRPV3-ARD structure demonstrates that the finger between repeats 3 and 4, including N242, is important for TRPV3 activity (Shi et al., 2013). Mutations of residues within this finger involved in the formation of hydrogen bonds altered the channel response to 2-APB. Given that hydroxylation within ARDs can contribute to the formation of additional hydrogen bonds (Kelly et al., 2009), hydroxylation by FIH might alter activity the conformation of this important loop.

The physiological response to hypoxia might have important implications for known TRPV3 channel functions. For example,

peripheral nociception is sensitive to hypoxia in animal models of diabetes (Fuchs et al., 2010), the skin is known to sense and respond to hypoxia (Boutin et al., 2008), and the role in thermosensation might be linked to the disrupted thermoregulation observed in FIH-deficient mice (Zhang et al., 2010), all of which might be mediated by FIH-dependent hydroxylation. The bioinformatics strategy also identified up to ten other TRP channels as potential substrates of FIH, including TRPA1 and TRPC1. Regulation of TRP channels by FIH might be a widespread mechanism of oxygen-dependent regulation.

Finally, these results provide a new avenue for potential therapeutic manipulation of a subset of TRP channels in human disease. They also have important implications for the development of novel inhibitors of 2-oxoglutarate-dependent dioxygenases, including FIH, for the treatment of ischaemic diseases (Nagel et al., 2010), which could have unexpected consequences on the activity of TRP channels.

MATERIALS AND METHODS

Plasmids

pcDNA3.1-FIH and pcDNA3.1-FIH-H199A, MBP-FIH (Lando et al., 2002a), pET32a-mNotch1-ARD and pET32a-mNotch1-ARD-N1945A/N2012A (Zheng et al., 2008) were as described previously. Human TRPV3 was amplified from cDNA (Open Biosystems, clone 8143869) and subcloned into pAdTrack-CMV for use in transient transfections and pEF-IRES-puro6 (Linke et al., 2007) for generation of stably expressing cells. A Myc-6his tag was inserted to generate pEF-Myc-6his-hTRPV3-IRES-puro6. AR 3–6 of human (h)TRPV3 (amino acids 212–364), mTRPV3 (amino acids 212–364) and hTRPV4 (amino acids 235–393) were amplified by PCR from cDNA clones (Open Biosystems, clones 8143869, 40047664 and 40125997) and cloned into pET-32a (Novagen). hTRPV3-N242A was generated by site-directed mutagenesis (Quikchange, Stratagene).

Cell culture

Cells were maintained as described previously (Linke et al., 2007; Scragg et al., 2008). Stable cell lines were generated using pEF-IRES-puro6-hTRPV3, pEF-IRES-puro6-hTRPV3-N242A and pEF-Myc-6his-hTRPV3-IRES-puro6 (Lando et al., 2002b; Scragg et al., 2008). HEK293T cells were transfected by using Polyfect (Qiagen) and HEK293 cells with Genejammer (Agilent Technologies).

CO_2 capture assays

Thioredoxin (Trx)-6his–hTRPV3-ARD3-6, Trx-6his–mTRPV3-ARD3-6, Trx-6his–hTRPV4-ARD3-6, Trx-6his–mNotch1-ARD1-6 and MBP–hFIH proteins were generated as described previously (Linke et al., 2007; Zheng et al., 2008). Hypoxia (1% oxygen) was achieved using an Edwards Instrument Hypoxia Workstation. All results were obtained from at least three independent experiments, each performed in triplicate.

In vitro pull-down assays

Recombinant Trx-6his-tagged TRPV proteins were purified and used for affinity pull-downs as described previously (Linke et al., 2007; Zheng et al., 2008). Western blotting was performed using an anti-FIH antibody (Novus Biologicals, NB100-428) as described previously (Linke et al., 2007; Zheng et al., 2008).

Mass spectrometry

Myc-6his–hTRPV3 was purified from stably expressing HEK293T cells or cells co-transfected with Myc-6his–hTRPV3 and FIH, using immobilised metal ion affinity adsorption. Purified protein was then separated by SDS-PAGE and subjected to in-gel tryptic digestion as described previously (Hastie et al., 2012; Peet et al., 2004). In-gel digests were subjected to capillary high performance liquid chromatography (HPLC) and either arrayed onto matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF)/TOF targets for MALDI-TOF/TOF-MS and -MS/MS (Dave et al., 2011) or sprayed directly into the ion-source of a High-Performance LTQ-Orbitrap Elite mass spectrometer essentially as described previously (Joubert et al., 2014). Quantification of hydroxylated versus nonhydroxylated peptides was calculated from the area under peaks in MS/MS.

Electrophysiology

Whole-cell patch clamping was performed using a computer-based patch-clamp amplifier (EPC-9, HEKA Electronics, Germany) and PULSE software (HEKA Electronics). The bath solution contained 140 mM NaCl, 4 mM CsCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.4, 22±1℃). The intracellular solution contained 120 mM caesium glutamate, 4 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM MgATP, 10 mM EGTA and 10 mM HEPES, pH 7.2. Patch pipettes were pulled from borosilicate glass with a resistance of 3–5 MΩ. Series resistance did not exceed 20 MΩ and was 60–70% compensated. To monitor changes in the amplitude of the membrane currents, voltage ramps between −120 and +120 mV were applied every 2 s, starting after achieving whole-cell configuration. Acquired currents were filtered at 2.7 kHz and sampled at 10 kHz. The holding potential was 0 mV throughout. Cell capacitance was compensated automatically by the EPC9 amplifier.

Acute hypoxia experiments

Cells were continuously perfused (3–5 ml/min, 22±1℃) with bath solution (as above) gassed either with air or 100% nitrogen, the latter resulting in a bath pO_2 of 20.2±1.7 mmHg (±s.e.m.; n=10) using a polarised carbon fibre electrode. Pipettes (3–6 MΩ) were filled with a solution containing 125 mM CsCl, 3 mM $CaCl_2$, 1 mM MgATP, 5 mM $MgCl_2$, 10 mM EGTA and 10 mM HEPES, pH 7.3. Series resistance was monitored throughout, and experiments were terminated if this varied by >20%. Signals were acquired using an Axopatch200B controlled by Clampex9.0 software through a Digidata 1322A interface (Axon Instruments, Inc., Foster City, CA). Data were filtered at 1 kHz and digitized at 5 kHz. To monitor ionic currents, voltage ramps were used as described above. Offline analysis was carried out using Clampfit9, and data are expressed as the mean±s.e.m.

Competing interests

The authors declare no competing interests.

Author contributions

S.K., M.L.W., C.P., J.J.G., J.M.G., G.Y.R. and D.J.P. conceived of and designed experiments. S.K., M.D., N.R.S., L.S., W.L.L., M.L.D., J.L.S., J.C., K.A.D. and D.J.P. performed experiments. S.K., M.D., N.S., L.S., W.L.L., M.L.D., J.L.S., J.C., K.A.D., M.L.W., C.P., J.J.G., J.M.G., G.Y.R. and D.J.P. analysed data. J.M.G., G.Y.R. and D.J.P. wrote the manuscript.

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