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Hypoxic induction of T-type Ca^{2+} channels in rat cardiac myocytes: role of HIF-1α and RhoA/ROCK signalling

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Key points

- T-type Ca^{2+} channels are expressed in the ventricular myocytes of the fetal and perinatal heart, but are downregulated as development progresses. However, these channels are re-expressed in adult cardiomyocytes under pathological conditions.
- Hypoxia induces the upregulation of the T-type Ca^{2+} channel $Ca_V3.2$ mRNA in cardiac myocytes, whereas $Ca_V3.1$ mRNA is not significantly altered.
- The effect of hypoxia on $Ca_V3.2$ mRNA requires hypoxia inducible factor-1α (HIF-1α) stabilization and involves the small monomeric G-protein RhoA and its effector ROCKI.
- Our results suggest that the hypoxic regulation of the $Ca_V3.2$ channels may be involved in the increased probability of developing arrhythmias observed in ischemic situations, and in the pathogenesis of diseases associated with hypoxic Ca^{2+} overload.

Abstract T-type Ca^{2+} channels are expressed in the ventricular myocytes of the fetal and perinatal heart, but are normally downregulated as development progresses. Interestingly, however, these channels are re-expressed in adult cardiomyocytes under pathological conditions. We investigated low voltage-activated T-type Ca^{2+} channel regulation in hypoxia in rat cardiomyocytes. Molecular studies revealed that hypoxia induces the upregulation of $Ca_V3.2$ mRNA, whereas $Ca_V3.1$ mRNA is not significantly altered. The effect of hypoxia on $Ca_V3.2$ mRNA was time- and dose-dependent, and required hypoxia inducible factor-1α (HIF-1α) stabilization. Patch-clamp recordings confirmed that T-type Ca^{2+} channel currents were upregulated in hypoxic conditions, and the addition of 50 μM $NiCl_2$ (a T-type channel blocker) demonstrated that the $Ca_V3.2$ channel is responsible for this upregulation. This increase in current density was not accompanied by significant changes in the $Ca_V3.2$ channel electrophysiological properties. The small monomeric G-protein RhoA and its effector Rho-associated kinase I (ROCKI), which are known to play important roles in cardiovascular physiology, were also upregulated in neonatal rat ventricular myocytes subjected to hypoxia. Pharmacological experiments indicated that both proteins were involved in the observed upregulation of the $Ca_V3.2$ channel and the stabilization of HIF-1α that occurred in response to hypoxia. These results suggest a possible role for $Ca_V3.2$ channels in the increased probability of developing arrhythmias observed in ischaemic situations, and in the pathogenesis of diseases associated with hypoxic Ca^{2+} overload.

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Abbreviations Ca_V channels, voltage-gated Ca^{2+} channels; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; DMOG, dimethyloxalylglycine; DPI, diphenyliodonium; HIF, hypoxia inducible factor; NMDG, *N*-methyl-D-glucamine; NRVMs, neonatal rat ventricular myocytes; P_{O_2}, partial pressure of oxygen; ROCK, Rho-associated kinase; ROS, reactive oxygen species; siRNA, small interfering RNA.

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Introduction

In the heart, voltage-dependent Ca^{2+} channels are involved in the regulation of essential cellular functions, which include excitability, contraction, hormonal secretion and possibly gene transcription (Ono & Iijima, 2010). Cardiac myocytes mainly express high voltage-activated L-type Ca^{2+} channels, which are responsible for Ca^{2+} influx into the cytoplasm during the action potential. In contrast, low voltage-activated T-type Ca^{2+} channels are expressed in cells of the nodes and the conduction system, thereby controlling heart rate. In the myocardium, T-type channels are expressed only during development and the perinatal period, but are absent in adults (Leuranguer *et al.* 2000; Ferron *et al.* 2002). Interestingly, however, they can be re-expressed in adult cardiomyocytes in certain pathological conditions (Nuss & Houser, 1993; Martinez *et al.* 1999; Izumi *et al.* 2003), although the mechanisms involved in this re-expression are not known (Vassort *et al.* 2006). Among the pathological conditions in which T-type Ca^{2+} channels have been reported to be re-expressed in the ventricle are those associated with ischaemia, such as myocardial infarction and heart failure (Huang *et al.* 2000; Izumi *et al.* 2003; Vassort *et al.* 2006). Hypoxia is one of the main components of ischaemia, and oxygen (O_2) is a major determinant of myocardial gene expression. As myocardial O_2 levels decrease, gene expression patterns in the heart are significantly altered (Huang *et al.* 2004). These molecular modifications are intended to reduce the cellular need and dependence on O_2 and increase O_2 supply (Lopez-Barneo *et al.* 2004). These cellular responses to chronic hypoxia are mainly due to the activity of different transcription factors, in particular the family of 'hypoxia inducible factors' (HIFs) (Bruick & McKnight, 2001; Semenza, 2001). An increase in the activity of these transcription factors leads to changes in the expression of multiple genes, including ion channels (Del Toro *et al.* 2003; Bautista *et al.* 2009; Ahn *et al.* 2012; Chu *et al.* 2012). However, the role of these ion channels in the adaptation to chronic hypoxia in the heart and other organs is currently a matter of debate.

Three different genes that code for T-type channels have been identified, $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$, the pore-forming subunits of which are α1G, α1H and α1I, respectively (Perez-Reyes, 2003). Only the $Ca_v3.1$ and $Ca_v3.2$ isoforms are expressed in the mammalian myocardium (Cribbs *et al.* 1998; Perez-Reyes *et al.* 1998). Chronic hypoxia increases $Ca_v3.2$ channel expression in PC12 (Del Toro *et al.* 2003), chromaffin (Carabelli *et al.* 2007) and pulmonary artery smooth muscle cells (Wan *et al.* 2013; and authors' unpublished observations), although the molecular mechanisms involved in this regulation are not fully understood. Similarly, it is not known whether or how $Ca_v3.2$ is regulated by hypoxia in cardiac myocytes. The small G protein RhoA and its main effectors, the Rho-associated kinases (ROCKs), are known to be important in cardiovascular physiology (Loirand *et al.* 2006; Nunes *et al.* 2010). Changes in the activity of these two proteins have been shown to contribute to some cardiac pathologies, including cardiac hypertrophy and heart failure (Miyamoto *et al.* 2010; Shi *et al.* 2011), in which T-type Ca^{2+} channels are also known to participate (Martinez *et al.* 1999; Chiang *et al.* 2009; Kuwahara & Nakao, 2011). Recent reports have demonstrated that hypoxia alters the expression and/or activity of RhoA and ROCK proteins in various cell types (Sauzeau *et al.* 2003; Turcotte *et al.* 2003; Bailly *et al.* 2004; Jin *et al.* 2006; Chi *et al.* 2010). It has also been shown that RhoA participates in the activation of transcription factors (Brown *et al.* 2006), including HIF-1α (Turcotte *et al.* 2003). However, the possible interaction between RhoA/ROCK and HIF-1α in cardiac myocytes, and their role in the regulation of $Ca_v3.2$ T-type channel expression have not been studied.

Here we report that chronic hypoxia upregulates expression of the $Ca_v3.2$ (α1H subunit) T-type Ca^{2+} channel in rat ventricular myocytes, and that this effect is mediated by the HIF-1α transcription factor. Our findings also demonstrate that RhoA activity is significantly increased under low oxygen conditions in cardiac myocytes, and that the RhoA/ROCK pathway is involved in the regulation of HIF-1α stabilization. Our results suggest that the hypoxic upregulation of the T-type Ca^{2+} channel could participate in pathophysiological processes such as cardiac hypertrophy and the increased probability of developing arrhythmias observed in ischaemic situations.

Methods

Ethical approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Seville, in compliance with the guidelines determined by the European legislation on animal experimentation.

Experimental animals

Wistar rats were maintained at 22°C on a 12–12 h light–dark cycle with free access to rodent chow and water. For hypoxia experiments, animals were maintained in a hypoxic chamber with a controlled atmosphere (O_2 control glove box for *in vivo* studies; COY Laboratory Products, Grass Lake, MI, USA) for variable periods of time.

Primary culture of rat ventricular myocytes

Neonatal rat ventricular myocytes (NRVMs) were prepared from 1- to 3-day-old animals and cultured as

Table 1. Primers for quantitative PCR experiments

Gene name	Forward primer	Reverse primer
Cav3.1	CAAGCTCCTGGAGACACAGAGTAC	CACTGTCTGCCTTGGAGCAA
Cav3.2	CTTCCTCAGCGTCTCCAACTACAT	AGGGCTACCACCTTCACCATC
Cav1.2	TCATCTTCAGCCCAAACAACAG	TTGGTGAAGATCGTGTCATTGAC
RhoA	GGATCTTCGGAATGATGAGCA	TGTTTGCCATATCTCTGCCTTCT
ROCKI	TATGAAGTAGTAAAGGTAATCGGCAGAG	CTGGTGGATTTATGCCTTACCAA
ROCKII	AATCAAATCAGCATCCTTCTTTAAGAAT	CTGGAGCTGCCGTCTCTCTTAT
18S rRNA	AACGAGACTCTGGCATGCTAACTA	GCCACTTGTCCCTCTAAGAAGTTG

described by Akao *et al.* (2001). Adult rat ventricular myocytes were prepared from 2-month-old males according to the protocol described by Martinez *et al.* (1999). For hypoxic treatments, cells were placed in a oxygen control incubator (Galaxy oxygen control incubator; RS Biotech Laboratory Equipment, Irvine, UK) that maintained a constant environment (5% CO_2 and either 1 or 3% O_2 balanced with N_2) for variable periods of time. Dimethyloxalylglycine (DMOG) was obtained from Frontier Scientific (Logan, UT, USA). C3 transferase was obtained from Cytoskeleton (Denver, CO, USA). All other reagents and chemicals were obtained from Sigma (St Louis, MO, USA).

Total RNA extraction and real-time RT-PCR

Total RNA was isolated using the Nucleospin II kit (Macherey-Nagel, Bethlehem, PA, USA) or the Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was generated using the Superscript II RNase H- RT kit (Invitrogen). Relative quantification of gene expression was performed by real-time PCR (qRT-PCR) using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) with the ABI Prism 7500 Sequence Detection System (Applied Biosystems). Cycle threshold (Ct) values were normalized to the Ct values of 18S rRNA, which was used as an endogenous gene. The sequences of the oligonucleotides used in the PCR reactions are provided in Table 1.

Western blotting

Cells were homogenized in standard lysis buffer using protease inhibitors. Protein concentrations were determined by the Lowry method. Lysates (50 μg) were resolved by 8% SDS-PAGE followed by transfer to Protein Blotting polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). Membranes were probed with different antibodies (see below) and developed with the enhanced chemiluminescence ECL plus Western blotting detection system (GE Healthcare, Piscataway, NJ, USA). Antibodies and dilutions used in the Western blots were α-tubulin (1:10 000, Sigma), RhoA (1:500, Cytoskeleton), ROCKI (1:500, BD Biosciences, Franklin Lakes, NJ, USA), ROCKII (1:500, BD Biosciences).

siRNA experiments and transfection studies

Cells were seeded 48–72 h prior to small interfering RNA (siRNA) or cDNA transfection. They were then transfected with the siRNAs using Lipofectamine RNAiMAX (Invitrogen) for 24–48 h in OptiMEM I Reduced Serum Medium (Gibco, Waltham, MA, USA). Cells were used or harvested at the time points indicated in the different experiments. siRNA sequences are indicated in Table 2, except for the scramble siRNA (Ambion, Austin, TX, USA). Transfection of mutated HIF-1α (kindly provided by Dr C. W. Pugh; Masson *et al.* 2001) was performed using Lipofectamine 2000.

Analysis of RhoA activity

The activity of RhoA was addressed using a biochemical assay that measures the amount of active, GTP-bound RhoA protein (G-LISA RhoA Activation Assay Biochem Kit (absorbance based); Cytoskeleton).

Electrophysiological recordings

Macroscopic currents were recorded using the whole-cell configuration of the patch-clamp technique as adapted in our laboratory (Bautista *et al.* 2009). Patch electrodes (2–3 MΩ) were pulled from borosilicate glass (Sutter Instruments, Novato, CA, USA; 1.5–1.6 mm), and fire-polished on an MF-830 microforge (Narishige, London, UK). Voltage-clamp recordings were obtained with an EPC-10 patch-clamp amplifier using standard voltage-clamp protocols designed with Pulse software (Heka Elektronik, Lambrecht/Pfalz, Germany). Unless otherwise noted, the holding potential was −90 mV. Data were filtered at 10 kHz, digitized at a sampling interval of 20 μs and stored on a Macintosh computer. Off-line analysis of data was performed using Pulse Fit (Heka Elektronik). All experiments were conducted at room temperature, 22–24°C. For whole-cell patch-clamp recordings in NRVMs, the internal solution contained

Table 2. Oligonucleotides used for siRNA experiments

Gene name	Sense	Antisense
HIF-1α	GCUUGCUCAUCAGUUGCCAtt	UGGCAACUGAUGAGCAAGCtt
HIF-2α	GGCCAAACAUGGAGGAUAUtt	AUAUCCUCCAAUGUUUGGCCtt

(in mM): 110 CsCl, 30 CsF, 10 EGTA, 10 Hepes and 4 Mg-ATP; the pH was adjusted to 7.2, and osmolality was 290 mosmol kg^{-1}. The standard bath solution contained (in mM): 140 NaCl, 9 $BaCl_2$, 1 $CaCl_2$, 10 Hepes and 10 glucose; the pH was adjusted to 7.4, and osmolality was 300 mosmol kg^{-1}. In some experiments, NaCl was substituted equimolarly with the impermeant *N*-methyl-D-glucamine (NMDG). For whole-cell patch-clamp recordings in adult cardiomyocytes, the internal solution contained (in mM): 100 CsCl, 20 TEA, 5 EGTA, 0.06 $CaCl_2$, 10 Hepes, 0.4 Na_2-GTP, 5 phosphocreatine and 5 Mg-ATP; the pH was adjusted to 7.2, and osmolality was 290 mosmol kg^{-1}. The standard bath solution contained (in mM): 140 choline-Cl, 5 CsCl, 0.5 $MgCl_2$, 2 4-aminopyridine, 5 $CaCl_2$, 5 Hepes and 5 glucose; the pH was adjusted to 7.4, and osmolality was 300 mosmol kg^{-1}. Nifedipine was obtained from Sigma.

Statistics

Unless otherwise specified, data were presented as mean ± standard error of the mean (SEM) with the number (n) of experiments indicated. Normality was tested with the Shapiro–Wilk test. Data from two groups were analysed with either t test or paired t test. Data with multiple groups were analysed with analysis of variance (ANOVA) or repeated-measures (RM) ANOVA followed by *post hoc* Tukey's test. A P value of < 0.05 was considered statistically significant.

Results

Hypoxic regulation of the $Ca_v3.2$ T-type channel expression in neonatal rat ventricular myocytes (NRVM)

It is known that in NRVMs two types of Ca^{2+} currents are generated upon depolarization: high voltage-activated (L-type) and low voltage-activated (T-type) Ca^{2+} currents. Figure 1*A* shows a representative family of Ca^{2+} currents recorded in a NRVM during 8 ms step depolarizations to variable membrane voltages. A current–voltage (*I–V*) curve of the averaged peak Ca^{2+} currents is represented in Fig. 1*B*. As expected, the predominant Ca^{2+} current was the robust high voltage-activated L-type Ca^{2+} current, but there was a small contribution of the low voltage-activated T-type current (see the small 'shoulder' (arrow) observed in the *I–V* graph at negative potentials). One of the conventional methods used to demonstrate the presence of T-type currents is the study of tail currents recorded at the end of the stimulus, as the deactivation kinetics of L- and T-type currents have different time courses (Armstrong & Matteson, 1985; Castellano & Lopez-Barneo, 1991; Levitsky & Lopez-Barneo, 2009). Analysis of the data revealed that the deactivating current was well fitted by a double, but not by a single, exponential function (Fig. 1*C*), which is consistent with the presence of two populations of Ca^{2+} channels in NRVMs. The average time constants of the exponential functions were 0.16 ms for the fast component and 1.69 ms for the slow component, values in accordance with those reported for L-type and T-type channels, respectively (Castellano & Lopez-Barneo, 1991; Klockner *et al.* 1999; McRory *et al.* 2001; Levitsky & Lopez-Barneo, 2009). Figure 1*D* shows the analysis of the contribution of the fast and slow components to the total tail current in NRVMs.

NRVMs express the $Ca_v1.2$ (α1C) L-type Ca^{2+} channel, and $Ca_v3.1$ (α1G) and $Ca_v3.2$ (α1H) T-type Ca^{2+} channels (Cribbs *et al.* 1998; Perez-Reyes *et al.* 1998). We sought to determine whether hypoxia regulates the expression of these channels in cultured NRVMs. Exposure of NRVMs to 1% O_2 for 24 h produced a selective increase (≈ 6-fold) in the $Ca_v3.2$ mRNA level, but no significant change in the mRNA levels of the other voltage-dependent Ca^{2+} channels expressed in these cells ($Ca_v3.1$ and $Ca_v1.2$; Fig. 1*E*). The effect of hypoxia on $Ca_v3.2$ mRNA showed a peak at 8 h and partial reversion after 48 h exposure to 1% O_2 (Fig. 1*F*). This effect was dose-dependent, as application of 3% O_2 for 24 h led to an ≈ 3-fold increase in the amount of $Ca_v3.2$ mRNA, whereas 1% O_2 (used in most of the experiments in this study) produced an ≈ 6-fold increase (Fig. 1*G*). Treatment of NRVMs with 1 mM DMOG, a hypoxia mimetic (see below), resulted in a similar increase in the $Ca_v3.2$ subunit mRNA level (Fig. 1*G*). Taken together, these results show that chronic hypoxia induces a marked and selective upregulation of the $Ca_v3.2$ T-type channel mRNA in cardiac NRVM myocytes.

Effect of hypoxia on T-type Ca^{2+} channel currents in NRVMs

The molecular data presented in the preceding section were complemented by electrophysiological analyses in

NRVMs exposed to either normoxia or hypoxia (1% O_2) for 24 h. T-type channels have a characteristic fast inactivation time course, which is completed in less than 50 ms. Therefore, comparison of the tail currents recorded on repolarization after pulses lasting either 5 or 50 ms can be used to reveal the presence of T-type channels (see Fig. 2*A* and *B*). In normoxic NRVMs, the slowly deactivating component of the tail current, present on repolarization after the short pulse (grey trace), was absent at the end of the long pulse (black trace; Fig. 2*A*). Exposure of NRVMs to hypoxia (1% O_2, 24 h) produced a clear increase in the contribution of the slow component to the tail current after the 5 ms pulse (Fig. 2*B*). As expected, this increase was not observed on repolarization after the long pulse, as only the L-type channels contribute to its tail current. Detailed analysis of the contribution of the fast and slow components to the tail current showed that in hypoxia the current density due to the slow component increased 2-fold (Fig. 2*C*, left), whereas there was no change in the density of the fast component (Fig. 2*C*,

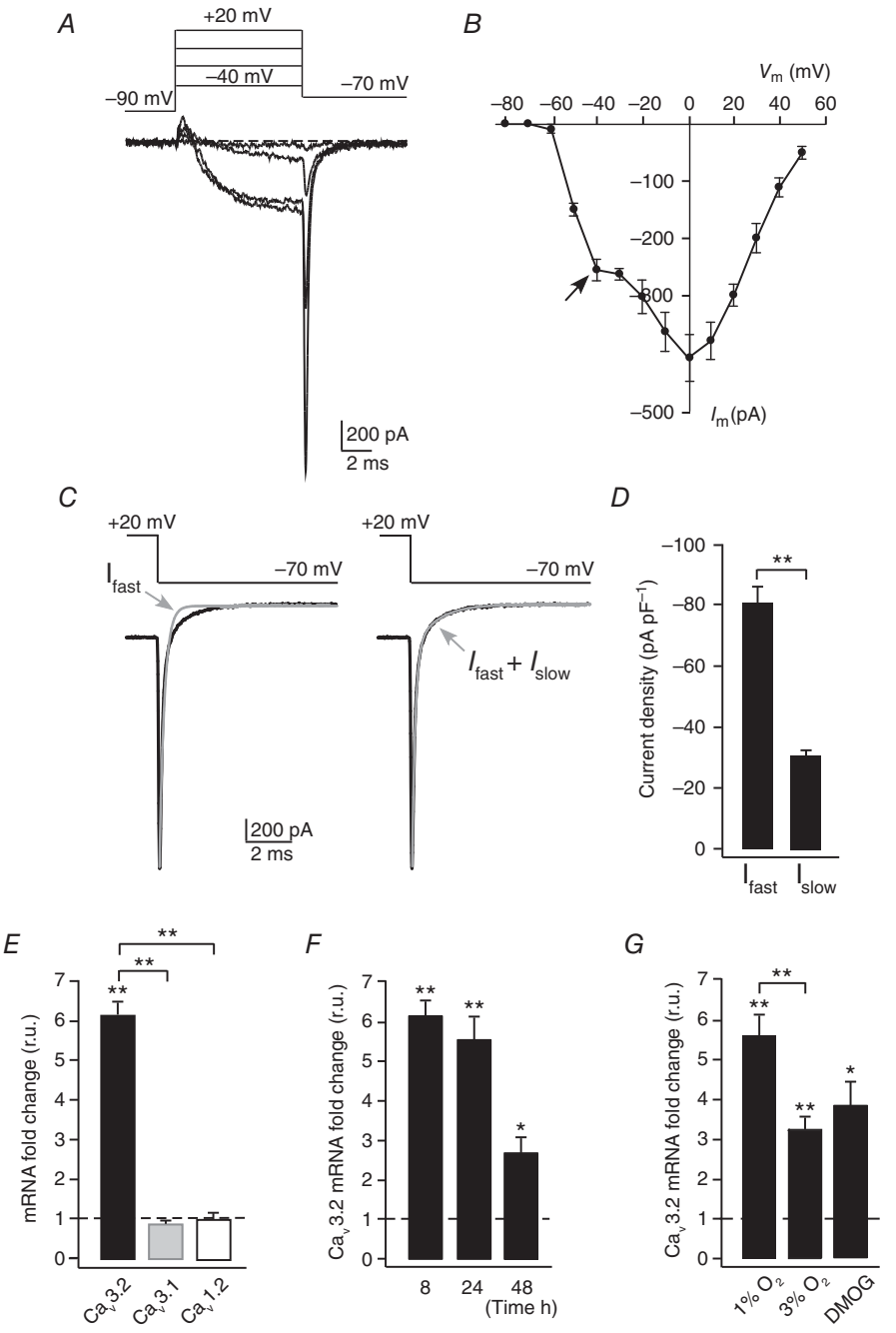

Figure 1. Hypoxic upregulation of $Ca_v3.2$ T-type Ca^{2+} channel in NRVMs
A, representative Ca^{2+} currents recorded in normoxic conditions from an NRVM subjected to whole-cell patch clamp in response to 8 ms depolarizing pulses to −40, −20, 0 and +20 mV, from a holding potential of −90 mV. The decay of the tail currents generated upon repolarization to −70 mV reflects the time course of closure of the channels that were open during the pulse. Note that although the tail current is fast, it has a clear slow component. To eliminate Na^+ currents, NaCl was substituted equimolarly with the impermeant NMDG. *B*, averaged Ca^{2+} current–voltage relationship for NRVMs exposed to normoxia ($n = 11$). Note the 'shoulder' (arrow) reflecting the contribution of the T-type Ca^{2+} channels. *C*, single (left) and double (right) exponential functions fitted to the Ca^{2+} tail current recorded from a representative NRVM. Note that a single exponential function does not properly fit the current decay. The time constants of the exponential functions were 0.16 ± 0.01 ms ($n = 22$) for the fast component and 1.69 ± 0.09 ms ($n = 22$) for the slow component. *D*, current densities for the fast and slow components of the tail currents measured at the moment of repolarization ($n = 22$). *E*, selective increase in $Ca_v3.2$ mRNA in NRVMs incubated under hypoxic conditions (24 h in 1% O_2, $n = 4$). *F*, time course of $Ca_v3.2$ mRNA upregulation induced in a 1% O_2 atmosphere ($n = 4$). *G*, dose dependency of the upregulation of $Ca_v3.2$ mRNA in hypoxia (24 h, $n = 4$) and effect of 1 mM DMOG (24 h, $n = 7$). All results are normalized to the normoxic levels of the mRNAs. $^*P < 0.05$; $^{**}P < 0.01$.

right). As a consequence, the ratio of the slow to the fast component of the tail current increased in response to hypoxia (Fig. 2*D*).

In addition to the analysis of tail currents, we also studied T-type currents recorded during depolarizing pulses in the presence of nifedipine to block L-type Ca^{2+} channels. Figure 3*A* shows representative T-type currents generated upon application of depolarizing pulses to −40 mV in a normoxic NRVM in the absence (C, control; R, recovery) or presence of 50 μM $NiCl_2$ (Ni). This protocol allowed us to measure the amplitude of the T-type currents in response to depolarizations at different membrane potentials, and to identify the $Ca_v3.2$ channel as responsible for the upregulation of the T-type currents observed after the hypoxic treatments. Figure 3*B* shows the increase (≈ 2-fold) of maximal T-type current density (pA pF^{-1}) induced by both hypoxia (1% O_2) or DMOG (1 mM) applied for 24–48 h. Interestingly, DMOG produced an increase in current amplitude similar to that elicited by hypoxia, corroborating that this drug has a similar potency as hypoxia in our experimental conditions. Upregulation of the T-type current by hypoxia and DMOG is further illustrated in Fig. 3*C* at various membrane potentials. To investigate whether the increase in the T-type current observed in hypoxia was due to the upregulation of the $Ca_v3.2$ channels, we analysed the sensitivity of the Ca^{2+} current to $NiCl_2$, as these channels are more sensitive to the cation than

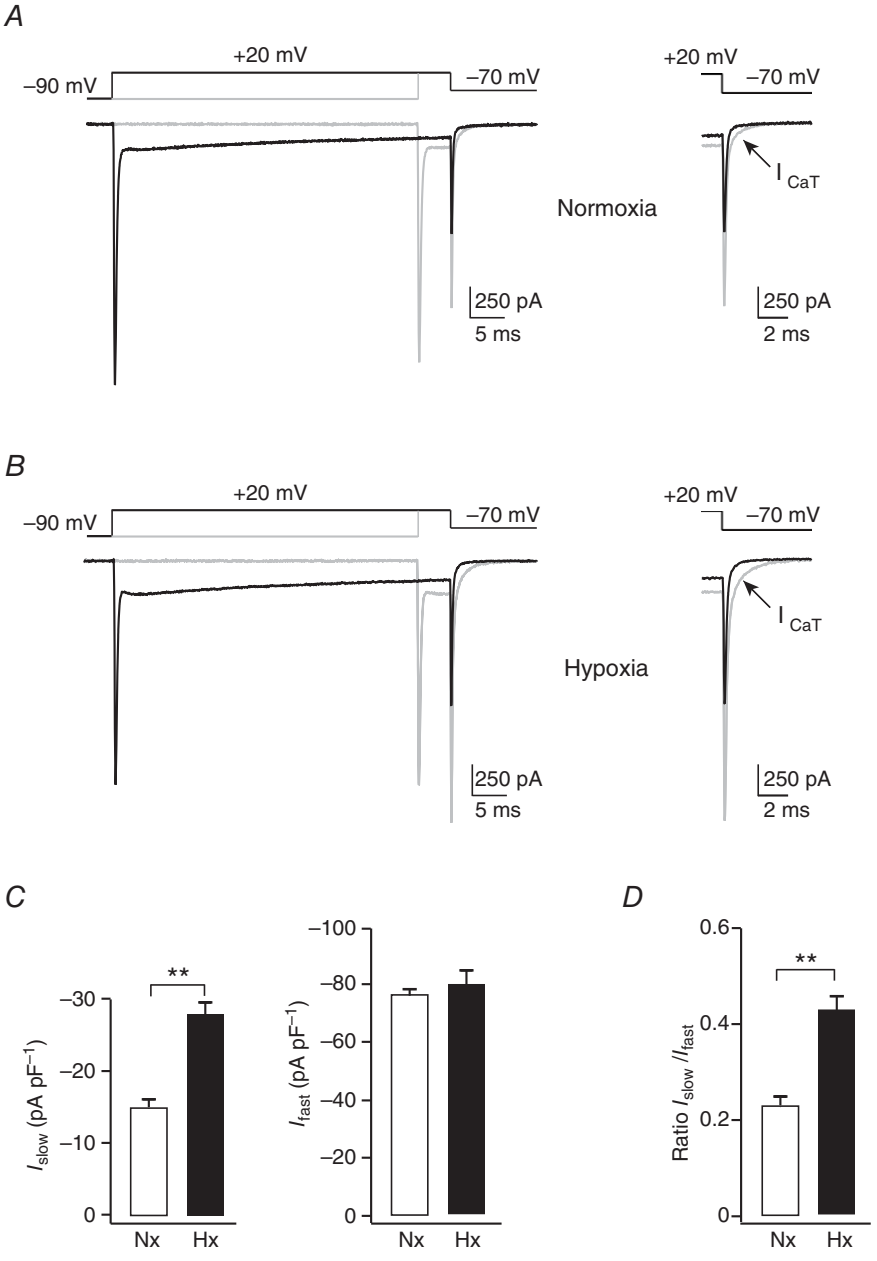

Figure 2. Increase of T-type Ca^{2+} currents in NRVMs exposed to chronic hypoxia
A and *B*, superposition of Na^+ and Ca^{2+} currents generated by depolarizing pulses of 5 ms (grey trace) and 50 ms (black trace) to +20 mV and upon return to −70 mV in normoxia (*A*) or hypoxia (*B*). The protocol is shown at the top of *A* and *B*. The tail currents are shown on an expanded time line to the right. The reduction in the amplitude of the slow component of the tail current (I_{CaT}) after the 50 ms pulse indicates inactivation of the T-type channels during the long-lasting depolarization. *C*, current density (pA pF^{-1}) of the slow (left) and fast (right) deactivating components of the Ca^{2+} currents in NRVM cultured in normoxia (Nx, 21% O_2) and hypoxia (Hx, 1% O_2) for 24 h. The slow component increased 2-fold (from −14.96 ± 0.19 pA pF^{-1} in normoxia ($n = 22$) to −28.05 ± 1.64 pA pF^{-1} in hypoxia ($n = 20$)), whereas the fast component current density was not affected (−76.62 ± 6.47 pA pF^{-1} ($n = 22$) in normoxia and −80.64 ± 5.49 pA pF^{-1} ($n = 20$) in hypoxia). *D*, ratio of current densities (slow/fast) calculated for each cell in the two experimental conditions. $^{**}P < 0.01$.

other subclasses of T-type channels. For example, in HEK-293 cells expressing recombinant proteins, $Ca_v3.2$ channels are 20 times more sensitive to $NiCl_2$ blockade (IC_{50} = 12 μM) than $Ca_v3.1$ channels (IC_{50} = 250 μM) (Lee *et al.* 1999). In our pharmacological experiments on ventricular myocytes we have used low (50 μM) and high (500 μM) concentrations of $NiCl_2$. In HEK-293 cells the low concentration blocks ≈70% of the $Ca_v3.2$ current and ≈20% of the $Ca_v3.1$ current, whereas the high concentration completely abolishes the $Ca_v3.2$ current, and ≈75% of the $Ca_v3.1$ current (Lee *et al.* 1999). The effects of 50 μM $NiCl_2$ on the T-type currents are illustrated

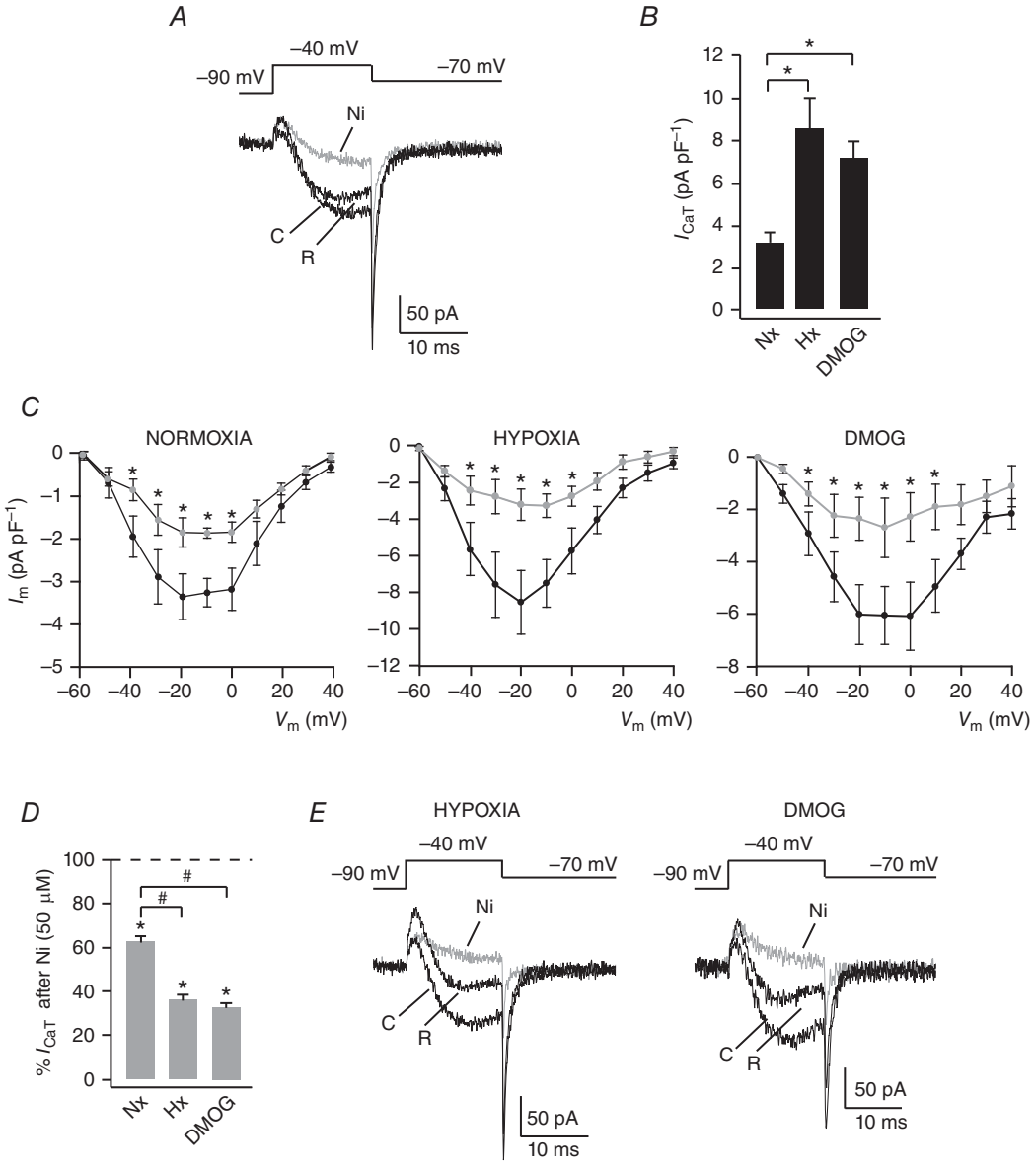

Figure 3. Effect of $NiCl_2$ on T-type Ca^{2+} currents in NRVM
A, protocol used to measure peak Ca^{2+} pulse currents and the effect of 50 μM $NiCl_2$ on T-type currents. Representative Ca^{2+} currents, generated during 15 ms pulses to −40 mV in an NRVM incubated in normoxia (C, control; R, recovery; Ni, 50 μM $NiCl_2$). *B*, upregulation of T-type currents (measured at −20 mV) after 48 h in hypoxia (Hx, 1% O_2) or DMOG (1 mM) (n = 5). *C*, averaged T-type Ca^{2+} current density–voltage relationships for NRVMs exposed to normoxia (left), hypoxia (centre) or DMOG (right) in the absence (black) or presence (grey) of 50 μM $NiCl_2$ (n = 5). Note the differences in the scales of the vertical axes. *D*, effect of 50 μM $NiCl_2$ on T-type current density measured at −20 mV in NRVMs exposed to normoxia (Nx), hypoxia (Hx) or DMOG (n = 5–6). $^*P < 0.05$ (control external solution *vs.* $NiCl_2$ treatment); $^\#P < 0.05$ (compared to normoxia). *E*, representative Ca^{2+} currents generated in NRVMs incubated in hypoxia (left) or in 1 mM DMOG (right) for 24–48 h in control external solution (C, control; R, recovery) or in the presence of 500 μM $NiCl_2$ (Ni). These experiments were all performed in the presence of 1 μM nifedipine in the external solution. $^*P < 0.05$.

by the current density–voltage relationships shown in Fig. 3*C*. NRVMs were incubated (24–48 h) in normoxia, hypoxia or with DMOG. The results clearly show that 50 μM $NiCl_2$ produced a stronger blockade of the currents in those cells incubated in hypoxia, as compared to normoxia. Furthermore, the effect of $NiCl_2$ on cells treated with DMOG was similar to that observed in hypoxic cells (Fig. 3*D*). Application of 500 μM $NiCl_2$ completely blocked the T-type currents in NRVMs (Fig. 3*E*). In the short term, the effect of 500 μM $NiCl_2$ was only partially reversible in most of the cells. Taken together, these electrophysiological data suggest strongly that, as suggested by the molecular studies (see Fig. 1*E*–*G*), the increase in the T-type current amplitude produced in hypoxia is due to the upregulation of $Ca_v3.2$ mRNA.

Effect of hypoxia on T-type Ca^{2+} channels in adult cardiac myocytes

Although expression of T-type channels in adult rat myocytes is negligible (Leuranguer *et al.* 2000; Ferron *et al.* 2002) (Fig. 4*A*), we sought to determine whether hypoxia can induce the re-expression of $Ca_v3.2$ channels, as it has been observed in some pathological conditions (Nuss & Houser, 1993; Martinez *et al.* 1999; Izumi *et al.* 2003). Exposure of adult rat cardiomyocytes in culture to 1% O_2 for 24 h produced a selective increase ($\approx$ 2-fold) in the $Ca_v3.2$ mRNA level, but no significant change in the $Ca_v3.1$ and $Ca_v1.2$ mRNAs (Fig. 4*B*). We were not able to determine the effect of longer exposures to hypoxia *in vitro*, as adult rat cardiomyocytes in culture did not resist sustained hypoxic exposures. In parallel with these *in vitro* experiments, we analysed the regulation of $Ca_v3.2$ mRNA *in vivo* using ventricular tissue obtained from adult rats exposed to hypoxia (9% O_2 for 24–48 h). In this preparation we also observed a strong ($\approx$4-fold) upregulation of $Ca_v3.2$ mRNA at 24 and 48 h (Fig. 4*C*). Similar to hypoxia, application of DMOG in adult rat cardiomyocytes in culture produced a $\approx$1.5-fold induction of $Ca_v3.2$ mRNA (Fig. 4*D*). These results confirmed that the hypoxic upregulation of $Ca_v3.2$ mRNA observed in NRVMs is also present in adult cardiomyocytes. Functional analyses of hypoxic upregulation of Cav3.2 channels could not be performed in adult cardiac myocytes because the protocol followed to prepare myocytes from animals previously exposed to hypoxia included a period of reoxygenation that masked the results. In addition, *in vitro* studies were hampered by the fact that after dissociation myocytes were rapidly deteriorated by the hypoxic treatment and therefore, although useful for molecular biology studies, did not yield stable patch clamp recordings. However, we were able to show a clear induction of T-type currents in dispersed myocytes treated with DMOG (Fig. 4*E*). The kinetic and pharmacological properties of the DMOG-induced current clearly indicate that it is mediated by $Ca_v3.2$ channels (Fig. 4*F*, *G*).

HIF-1α is involved in hypoxic $Ca_v3.2$ mRNA upregulation

To study the mechanisms underlying the effect of hypoxia on $Ca_v3.2$ channel expression, we first analysed $Ca_v3.2$ mRNA stability using the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). Application of DRB (200 μM) in high (normoxic) and low (hypoxic) O_2 tension resulted in a progressive decrease in the amount of $Ca_v3.2$ mRNA. However, the time course of mRNA degradation was unaffected by O_2 tension (Fig. 5*A*). These results suggest that changes in $Ca_v3.2$ mRNA stability are not responsible for the observed mRNA regulation, indicating that the effect of hypoxia is produced at the transcriptional level.

It has been proposed that HIF proteins are involved in the hypoxic regulation of the $Ca_v3.2$ subunit in PC12 cells (Del Toro *et al.* 2003). The involvement of HIF is supported by the presence of highly conserved hypoxia-responsive elements in the 5′-flanking region of the α1H gene (Del Toro *et al.* 2003; Sellak *et al.* 2014). We have shown in previous studies that both HIF-1α and HIF-2α proteins are expressed in cultured NRVMs, and that they are stabilized by hypoxia (Bautista *et al.* 2009). We have also reported that application of DMOG (1 mM), an inhibitor of prolyl hydroxylases that prevent hydroxylation and proteasomal degradation of HIF isoforms, resulted in the stabilization of the HIF-1α and HIF-2α proteins to a similar degree to that produced by exposure to hypoxia (Bautista *et al.* 2009). As shown above (Fig. 1*G*), treatment of NRVMs with DMOG induced a significant increase in the $Ca_v3.2$ subunit mRNA level. These results suggest strongly that HIF proteins are involved in the hypoxic upregulation of $Ca_v3.2$ mRNA.

To further explore the involvement of HIF in the hypoxic $Ca_v3.2$ mRNA upregulation, we exposed cells to hypoxia once they had been pre-incubated with siRNA against HIF-1α or HIF-2α (Fig. 5*B*; the protocol is detailed in the figure legend). Incubation of NRVMs with either HIF-α siRNA resulted in a marked (50–60%) and selective decrease in the corresponding mRNA, but did not affect $Ca_v3.2$ mRNA (Fig. 5*C*). We have previously shown that this decrease in HIF mRNAs is accompanied by a corresponding decrease in their protein levels (Bautista *et al.* 2009). We therefore tested the effect of HIF inhibition on $Ca_v3.2$ mRNA under hypoxic conditions. Inhibition of HIF-1α with its siRNA partially reversed the effect of hypoxia on $Ca_v3.2$ mRNA, whereas treatment with HIF-2α siRNA had no effect (Fig. 5*D*). A scrambled siRNA did not affect the HIF-1α, HIF-2α or $Ca_v3.2$ mRNA. To confirm the HIF-1α-dependent upregulation of $Ca_v3.2$ mRNA,

we performed transfection experiments using an HIF-1α double mutant that has constitutive activity (Masson *et al.* 2001). Expression of the plasmid HIF-1α-mut in normoxic conditions produced stabilization of the HIF-1α protein and induced the selective expression of $Ca_v3.2$ mRNA (Fig. 5*E*). Together, these data indicate that the upregulation of $Ca_v3.2$ mRNA expression by low O_2 tension is selectively mediated by HIF-1α.

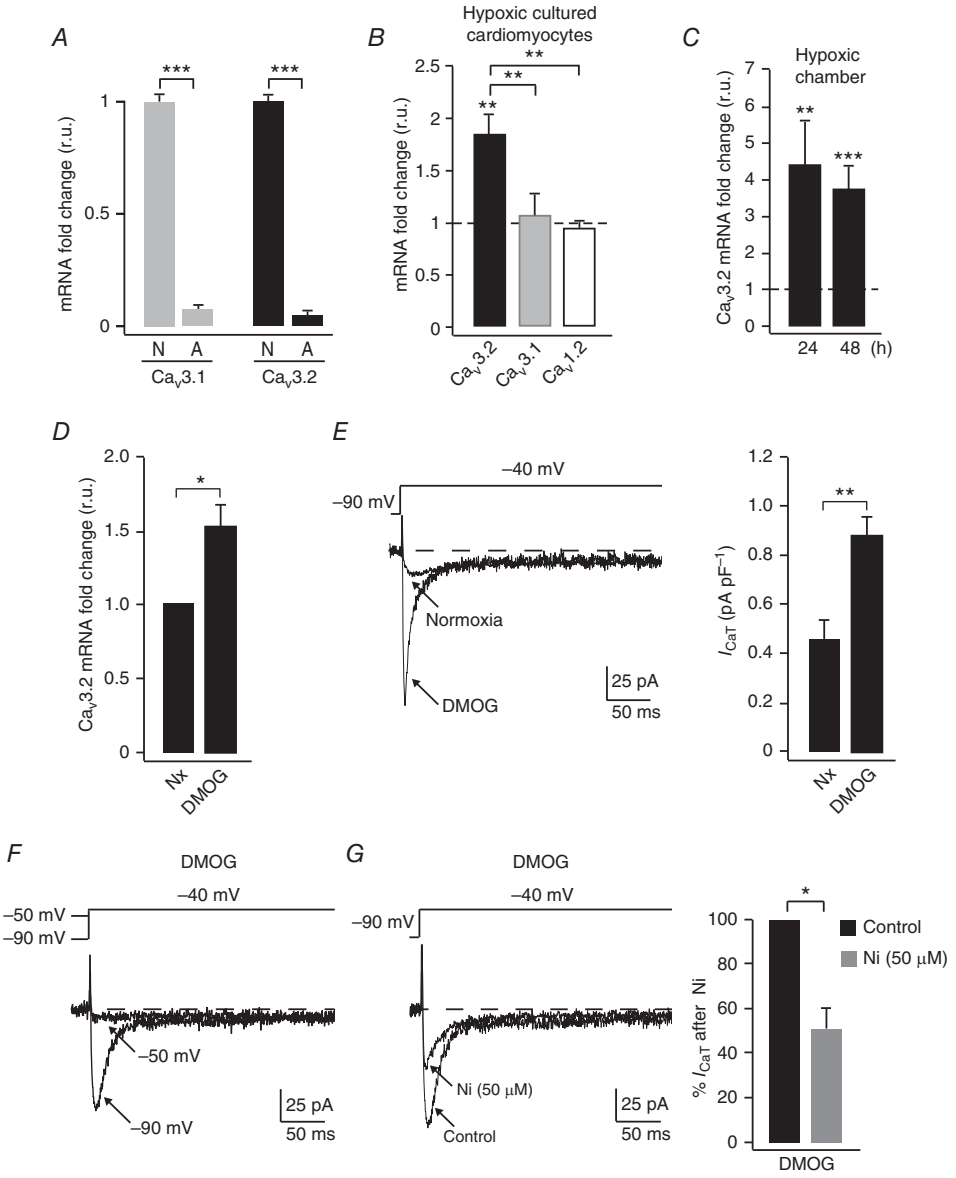

Figure 4. Hypoxic upregulation of $Ca_v3.2$ T-type Ca^{2+} channel in adult cardiac myocytes
A, relative expression of Ca_v3 mRNAs in neonatal (N) and adult (A) rat ventricular tissue ($n = 3$). *B*, selective upregulation of $Ca_v3.2$ mRNA in adult cardiomyocytes in culture subjected to hypoxia (24 h in 1% O_2, $n = 3$). *C*, upregulation of $Ca_v3.2$ mRNA in the ventricles of rats subjected to 9% hypoxia for 24 or 48 h in a hypoxic chamber ($n = 4$). *D*, effect of 1 mM DMOG on $Ca_v3.2$ mRNA in adult cardiomyocytes in culture (48 h, $n = 5$). *E*, representative T-type Ca^{2+} currents generated in adult cardiac myocytes incubated in normoxia (Nx) or in the presence of 1 mM DMOG for 48 h (left). Note the increased T-type current in the cell incubated in DMOG. Statistical analysis of the peak current density measured at −40 mV in normoxia or DMOG ($n = 5$, right). *F*, representative currents showing the effect of changes in the holding potential on T-type currents elicited at −40 mV in an adult cardiomyocyte incubated in the presence of 1 mM DMOG for 48 h. *G*, effect of 50 μM $NiCl_2$ on T-type currents elicited at −40 mV in a DMOG-treated adult cardiomyocyte (left). Statistical analysis of the effect of 50 μM $NiCl_2$ on T-type currents (right, $n = 3$). All results are normalized to the normoxic levels of the mRNAs. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Role of RhoA/ROCK in the hypoxic regulation of $Ca_V3.2$ mRNA

The small G protein RhoA and its main effectors, ROCKI and ROCKII, are expressed in the heart (Nakagawa *et al.* 1996). Hypoxia alters the expression and/or activity of RhoA and ROCK proteins in various cell types (Sauzeau *et al.* 2003; Turcotte *et al.* 2003; Bailly *et al.* 2004; Jin *et al.* 2006; Chi *et al.* 2010). However, the effect of hypoxia on RhoA/ROCK in cardiac myocytes has not been studied. We therefore queried whether the RhoA/ROCK pathway is regulated by hypoxia in NRVMs. Exposure of NRVMs to 1% O_2 for 4–24 h did not produce any significant effect on RhoA mRNA (Fig. 6*A*), but increased its protein level between 4 and 8 h (Fig. 6*B*). To interact with their effectors Rho proteins must be activated by binding to GTP. To assess the activation state of RhoA, we used an ELISA-based assay that measures the level of GTP-bound RhoA. Exposure of NRVMs to hypoxia (4 h) induced a significant increase in RhoA activity

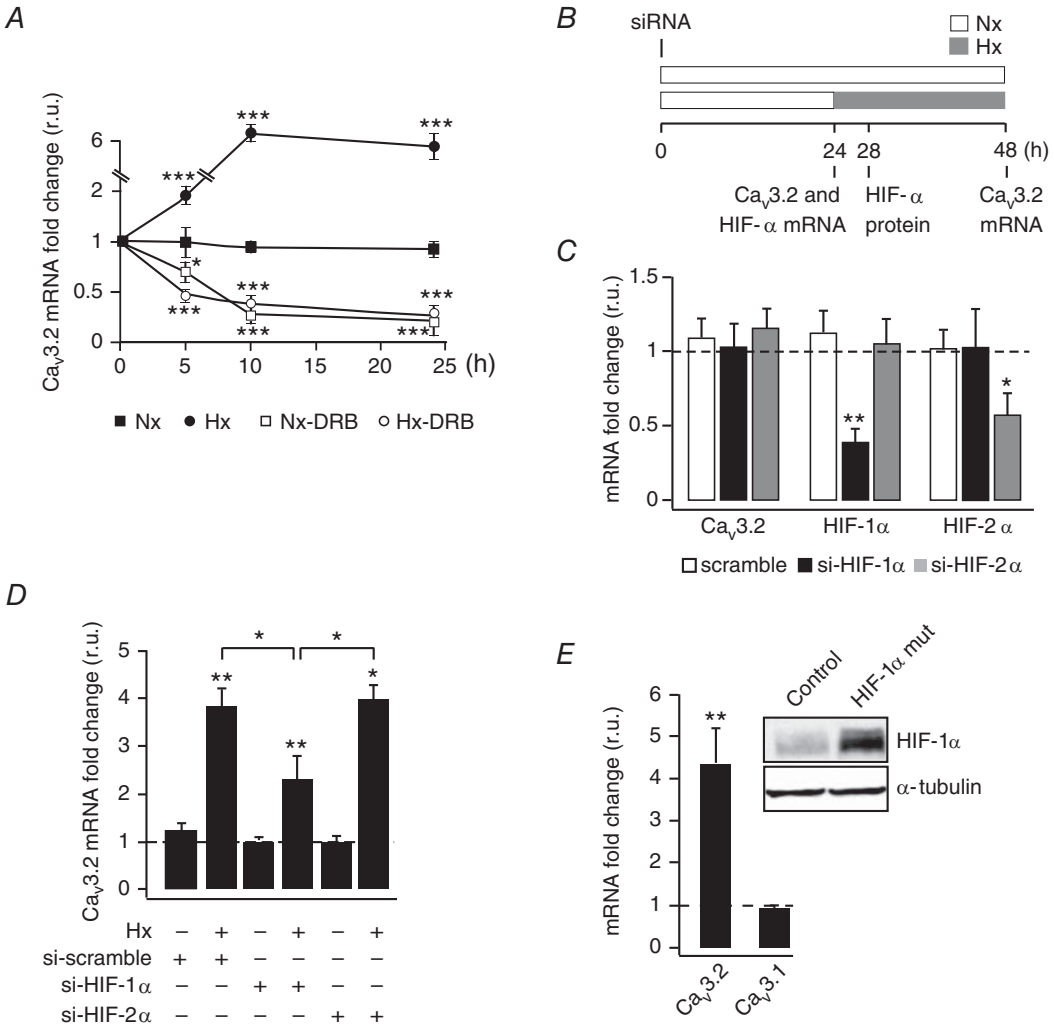

Figure 5. Role of HIF-1α in $Ca_V3.2$ mRNA hypoxic upregulation in NRVMs
A, study of $Ca_V3.2$ mRNA stability in NRVMs under normoxic (Nx) and hypoxic (Hx) conditions and in the absence or presence of 200 μM DRB ($n = 5$). *B*, scheme of the protocol used in the siRNA experiments. Samples were pre-incubated for 24 h with the corresponding HIF siRNAs (25 nM), at which point HIF-1α, HIF-2α and $Ca_V3.2$ mRNA levels were analysed by qRT-PCR. Scrambled siRNA was used as a negative control at the same concentration. Hypoxia (1% O_2) was then applied, and 4 h later, the levels of HIF-1α and HIF-2α proteins were analysed by Western blot. Twenty-four hours after application of hypoxia, $Ca_V3.2$ mRNA was measured. *C*, effect of the different siRNAs on HIF and $Ca_V3.2$ mRNAs before application of hypoxia ($n = 9$). *D*, $Ca_V3.2$ mRNA levels were analysed by qRT-PCR at 24 h in normoxia or hypoxia (Hx, 1% O_2) in the presence or absence of the HIF siRNAs ($n = 11$). *E*, effect of the overexpression of a constitutive form of HIF-1α (HIF-1α-mut) on the T-type Ca^{2+} channel isoforms expressed in NRVMs ($n = 5$). The inset shows the overexpression of the HIF-1α-mut protein in normoxia 24 h after transfection. All results are normalized to the normoxic levels of mRNA. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(Fig. 6*C*). Similar to RhoA, exposure of cardiac myocytes to hypoxia did not affect the mRNA level of ROCKI (Fig. 6*D*), but upregulated its protein level (Fig. 6*E*). In contrast, hypoxia did not affect the ROCKII mRNA (data not shown) or protein level (Fig. 6*F*). These results indicate, for the first time, that hypoxia induces the upregulation and activation of the RhoA/ROCKI pathway in NRVMs.

In light of these results, we were interested in identifying the upstream events that trigger the upregulation of RhoA in response to hypoxia. Reactive oxygen species (ROS) have been proposed as possible sensors involved in low O_2 detection (Chandel *et al.* 1998), and it is known that hypoxia stimulates the production of ROS in cardiac myocytes (Ngoh *et al.* 2011). It has also been shown that ROS are involved in the upregulation of RhoA during hypoxia in renal cell carcinoma (Turcotte *et al.* 2003). To determine whether ROS production also mediates the increase in RhoA activity observed in cardiomyocytes subjected to hypoxia, RhoA activity was studied in the presence of an ROS production inhibitor, diphenyliodonium (DPI). Addition of 10 μM DPI did not produce any effect on RhoA activity in normoxia, but completely prevented the upregulation observed in hypoxia (Fig. 7*A*), supporting the hypothesis that ROS production is involved in the increase in RhoA activity observed in NRVMs subjected to hypoxia.

As the RhoA/ROCK pathway is regulated by hypoxia and can alter HIF-1α protein levels (Turcotte *et al.* 2003; Hayashi *et al.* 2005; Takata *et al.* 2008), we next sought to study the possible interaction between RhoA/ROCK and HIF-1α in NRVMs. Application of a cell-permeable Rho inhibitor (C3 transferase) partially blocked HIF-1α stabilization in cardiac myocytes exposed to hypoxia for 4 h (Fig. 7*B*), indicating that in our preparation Rho activity modulates HIF-1α stabilization.

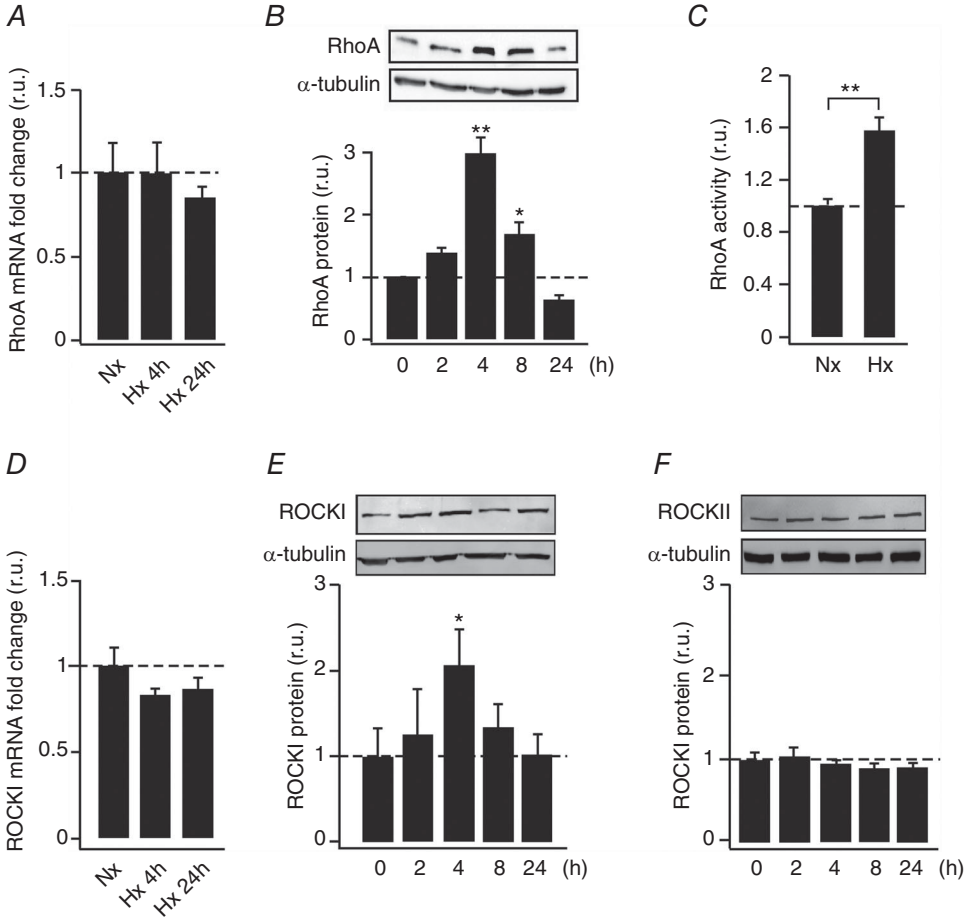

Figure 6. Regulation of the RhoA/ROCK pathway by hypoxia in NRVMs
A, lack of effect of hypoxia (Hx, 4 h and 24 h) on RhoA mRNA ($n = 8$). *B*, time course of the effects of hypoxia on RhoA protein, analysed by Western blot ($n = 7$). *C*, increase in RhoA activity measured, after 4 h in hypoxia, with a biochemical assay ($n = 5$). *D*, lack of significant effect of hypoxia (Hx, 4 h and 24 h) on ROCKI mRNA ($n = 6$). *E* and *F*, time course of the effects of hypoxia on ROCKI (*E*) and ROCKII (*F*) proteins, analysed by Western blot ($n = 8$). ROCKI protein was markedly increased after 4 h in hypoxia. All results are normalized to the normoxic levels of mRNA or protein. $^*P < 0.05$; $^{**}P < 0.01$. The hypoxic stimulus was 1% O_2.

To test if HIF-1α stabilization affects RhoA activity, we used the constitutive mutant HIF-1α-mut. Expression of HIF-1α-mut in normoxia did not affect RhoA activity in NRVMs (Fig. 7*C*). These data indicate that Rho proteins participate in the regulation of HIF-1α in hypoxia, but that stabilization of HIF-1α does not affect RhoA activity, suggesting that RhoA acts upstream of HIF-1α.

The preceding results show that in NRVMs RhoA regulates HIF-1α and that this transcription factor is responsible for the hypoxic upregulation of the $Ca_v3.2$ T-type Ca^{2+} channel. We therefore queried whether the RhoA/ROCK pathway is involved in this upregulation. To investigate this, we adopted a pharmacological approach. Application of the Rho inhibitor C3 transferase (2 μg ml^{-1}) or the ROCK inhibitors Y27632 (2.5 μM) and fasudil (10 μM) significantly abolished the hypoxic upregulation of $Ca_v3.2$ mRNA (Fig. 7*D*), suggesting involvement of the RhoA/ROCK pathway in this process. As RhoA is involved in $Ca_v3.2$ expression, we analysed the effect of ROS inhibition on T-type Ca^{2+} channel upregulation. Application of 10 μM DPI significantly blocked the effect of hypoxia on $Ca_v3.2$ mRNA (Fig. 7*E*), indicating that ROS production during hypoxia participates in upregulation of the $Ca_v3.2$ T-type Ca^{2+} channel. Taken together, these data indicate that the RhoA/ROCK pathway is modulated by low

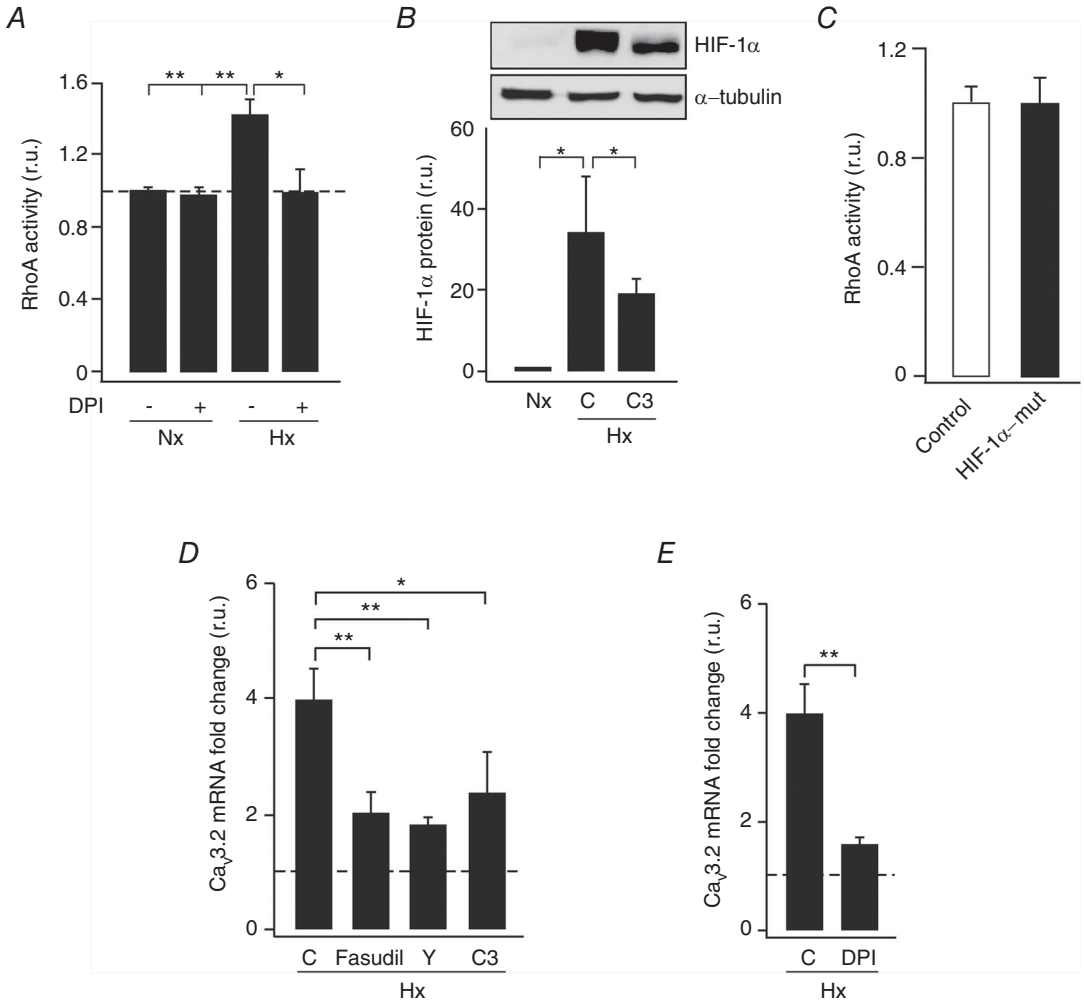

Figure 7. Cross-talk between RhoA/ROCK and HIF-1α in hypoxia-induced $Ca_v3.2$ mRNA upregulation
A, complete blockade of the hypoxia-induced increase in RhoA activity in the presence of 10 μM DPI, an ROS production inhibitor ($n = 5$). RhoA activity was measured after 4 h in hypoxia (1% O_2). *B*, effect of the Rho inhibitor C3 transferase on HIF-1α protein stabilization in hypoxic conditions ($n = 7$). Note that C3 transferase reduces HIF-1 protein stabilization induced by hypoxia (1% O_2, 4 h). C denotes control. *C*, effect of the overexpression of a constitutive form of HIF-1α on RhoA activity, measured with a biochemical assay 24 h after transfection ($n = 3$). *D*, effects of C3 transferase (2 μg ml^{-1}, $n = 4$) and ROCK inhibitors (10 μM fasudil ($n = 6$) and 2.5 μM Y27632 ($n = 6$)) on $Ca_v3.2$ mRNA hypoxic (1% O_2, 24 h) upregulation. *E*, reduction of $Ca_v3.2$ mRNA upregulation induced by hypoxia (1% O_2, 24 h) in the presence of 10 μM DPI ($n = 5$). All results are normalized to the normoxic levels of mRNA or protein. $^*P < 0.05$; $^{**}P < 0.01$.

O_2 tension in NRVMs, and participates in the hypoxic upregulation of cardiac $Ca_v3.2$ expression.

Discussion

The main findings in this study are: (1) chronic hypoxia upregulates expression of the $Ca_v3.2$ (α1H subunit) T-type Ca^{2+} channel in rat ventricular myocytes; (2) this effect is mediated by the HIF-1α transcription factor; (3) RhoA activity is significantly increased under low oxygen conditions in cardiac myocytes; and (4) the RhoA/ROCK pathway is involved in the regulation of HIF-1α stabilization.

Upregulation of the $Ca_v3.2$ Ca^{2+} channel by low O_2 tension in cardiac myocytes

T-type Ca^{2+} channels are expressed in the myocardium in the pre- and perinatal periods, but their expression level in adult myocytes is negligible (Leuranguer *et al.* 2000; Ferron *et al.* 2002). However, they are re-expressed in several pathophysiological conditions, some of which are associated with hypoxic conditions (Huang *et al.* 2000). Hypoxia upregulates the $Ca_v3.2$ channel in several cell types, including PC12 (Del Toro *et al.* 2003), chromaffin (Carabelli *et al.* 2007) and pulmonary artery smooth muscle cells (Wan *et al.* 2013; authors' unpublished observations), although the molecular mechanisms underlying this upregulation are not completely known. Here, we show that chronic hypoxia selectively upregulates $Ca_v3.2$ mRNA and current in NRVMs and adult cardiomyocytes. The effect of hypoxia on $Ca_v3.2$ mRNA was dose- and time-dependent, such as occurs with other genes whose expression is modulated by O_2 tension (Semenza, 1999; Bautista *et al.* 2009). Most of our experiments have been performed in 1% O_2 as it is a stimulus widely used in *in vitro* gene regulation studies, and considered as moderate hypoxia (Silverman *et al.* 1997; Hammond *et al.* 2014). In addition, this value is in the range of the expected interstitial fluid P_{O_2} level of rats maintained in an atmosphere with 9% O_2, the stimulus used in our *in vivo* studies.

Electrophysiological recordings obtained from NRVMs exposed to normoxia or hypoxia (1% O_2; 24 h) indicated that the T-type Ca^{2+} channel current is also upregulated in low O_2 conditions, representing the functional consequences of the results obtained in our molecular studies. This upregulation was due to an increase in the density of the $Ca_v3.2$ current, based on the following. (1) Analysis of the amplitude of the tail and pulse currents: comparison of the tail currents obtained after short (5 ms) and long (50 ms) depolarizing pulses confirmed the presence of two components with different inactivation and deactivation kinetics (Fig. 2). The slow component had a deactivation time constant of 1.69 ms, consistent with expression of the $Ca_v3.2$ channel (McRory *et al.* 2001; Levitsky & Lopez-Barneo, 2009). Analysis of the tail currents recorded after 5 ms pulses in NRVMs demonstrated that the amplitude of the slow component of deactivation almost doubled after 24 h in hypoxia (without changes in the time constant), suggesting that hypoxia up-regulates $Ca_v3.2$ channel proteins in the plasma membrane. Similar effects were observed when the T-type Ca^{2+} currents were studied in the presence of nifedipine, to eliminate the L-type current, and their amplitude was measured during the pulse (Fig. 3). The amplitude of the T-type current in cells subjected to hypoxia increased $\approx$ 2-fold, a value similar to that obtained when the tail currents were analysed. (2) The effect of $NiCl_2$: although both $Ca_v3.1$ and $Ca_v3.2$ channels are sensitive to $NiCl_2$, $Ca_v3.2$ is 20 times more sensitive than $Ca_v3.1$ to Ni^{2+}. Using recombinant channels expressed in HEK-293 cells, an IC_{50} of 12 μM for the $Ca_v3.2$ channel and an IC_{50} of 250 μM for the $Ca_v3.1$ channel have been reported (Lee *et al.* 1999). Our experiments showed that application of 50 μM $NiCl_2$ inhibited $\approx$40% of the T-type current generated in NRVMs incubated in normoxia. This result is consistent with that reported previously (Ferron *et al.* 2002), showing that in basal conditions, in 1- to 5-day-old rats the number of mRNA copies is higher for $Ca_v3.1$ than for the $Ca_v3.2$ channels ($\approx$60:40 ratio for $Ca_v3.1$ *vs.* $Ca_v3.2$ mRNAs). These authors also observed that the main contributor to the T-type current in basal conditions was the $Ca_v3.1$ channel. In our preparation, the normoxic mRNA level is 20% higher for $Ca_v3.1$ as compared to $Ca_v3.2$ (data not shown). This result, together with the effect of Ni^{2+} (blocking only $\approx$40% of the T-type current), indicates that $Ca_v3.2$ channels are not the main contributors to the T-type current in basal conditions (normoxia). Importantly, 50 μM $NiCl_2$ inhibited $\approx$70% of the current generated in the cells subjected to hypoxia or treated with DMOG. As 50 μM $NiCl_2$ is expected to block $\approx$70% of the $Ca_v3.2$ current (and $\approx$20% of the $Ca_v3.1$ current), these results confirm that the channel responsible for the hypoxia-enhanced T-type current is the $Ca_v3.2$ isoform.

The upregulation of the $Ca_v3.2$ channel in response to hypoxia observed in neonatal myocytes seemed to be also present in adult cardiomyocytes. A strong induction ($\approx$4-fold) of $Ca_v3.2$ mRNA has been observed in ventricles from adult rats exposed to 9% O_2 for 24–48 h. In addition, hypoxia and DMOG also upregulated $Ca_v3.2$ mRNA in cultured adult cardiomyocytes ($\approx$1.8- and $\approx$1.5-fold, respectively). Importantly, DMOG induced the re-expression of T-type currents in adult cardiomyocytes in culture (Fig. 4). Together, these results indicate that the signalling pathway regulating $Ca_v3.2$ mRNA expression in response to hypoxia is functional in adult cardiomyocytes.

Together, these data suggest that the enhanced contribution of the low threshold and slow deactivating component to the Ca^{2+} current in hypoxia reflects the upregulation of $Ca_v3.2$ protein, as a consequence of the increase in the amount of $Ca_v3.2$ mRNA observed in low O_2 conditions. Our observations differ from those of a report showing the effect of hypoxia/reoxygenation on T-type channels in NRVMs (Pluteanu & Cribbs, 2009). In that study, the authors showed that the $Ca_v3.2$ channel current is regulated by hypoxia, whereas its mRNA level is not altered. They also reported that $Ca_v3.1$ mRNA expression and the channel current were repressed in NRVMs subjected to hypoxia. However, we did not observe any significant effect of hypoxia on $Ca_v3.1$ mRNA. The differences between the two studies may be due to the way in which hypoxia was applied, i.e. an anaerobic microbiological system (Pluteanu & Cribbs, 2009) *versus* an incubator with a controlled and constant atmosphere (this study).

Mechanisms of $Ca_v3.2$ mRNA upregulation by hypoxia in NRVMs: dependence on HIF-1α stabilization

HIF proteins are highly expressed in cardiac tissue, where they play a pivotal role in the transcriptional response to changes in oxygen availability (Cai *et al.* 2008; Semenza, 2011). Our results suggest that hypoxia-dependent $Ca_v3.2$ channel upregulation is due to an increase in transcription, as the stability of its mRNA is not affected by O_2 tension. They also indicate that the effect of hypoxia is mimicked by DMOG, an inhibitor of prolyl hydroxylases (Semenza, 1999; Ivan *et al.* 2001; Jaakkola *et al.* 2001; Lopez-Barneo *et al.* 2001), suggesting a role for HIF proteins. Our siRNA experiments to selectively inhibit HIF-1α or HIF-2α directly demonstrate that HIF-1α is required for the upregulation of the $Ca_v3.2$ gene in hypoxia. Moreover, constitutive expression of HIF-1α in normoxia selectively induces $Ca_v3.2$ channel expression. This regulation is consistent with the presence of highly conserved hypoxia-responsive elements in the 5′-flanking region of the α1H gene (Del Toro *et al.* 2003; Sellak *et al.* 2014). Both Del Toro *et al.* (2003) and Carabelli *et al.* (2007) suggest that HIF proteins are implicated in the hypoxic regulation of $Ca_v3.2$ in PC12 and chromaffin cells, respectively. However, whereas HIF-2α seems to mediate the effect of hypoxia in PC12 cells (Del Toro *et al.* 2003), HIF-1α is responsible for this effect in NRVM.

Role of the RhoA/ROCK pathway in HIF-1α stabilization and $Ca_v3.2$ mRNA upregulation

In this study we show that the protein level and activity of RhoA are upregulated by chronic hypoxia in NRVMs. Several reports have documented the regulation of RhoA by hypoxia in different cell types (Sauzeau *et al.* 2003; Turcotte *et al.* 2003; Bailly *et al.* 2004; Jin *et al.* 2006). The increase in RhoA protein level and activity in NRVMs was time-dependent, showing a maximum after 4 h of hypoxic treatment. This time course of RhoA upregulation is similar to that observed in a hypoxic myocardial cell model (Huang *et al.* 2014) and in renal cell carcinoma (Turcotte *et al.* 2003). Moreover, ROCKI protein, the main RhoA effector, was also upregulated in NRVM. A modest increase in ROCKI has also been observed in breast cancer cell lines (Gilkes *et al.* 2014) and the myocardial cell model (Huang *et al.* 2014). It has been shown that hypoxia stimulates ROS production in different cell types, including cardiac myocytes (Ngoh *et al.* 2011), and that ROS are involved in the upregulation of RhoA during hypoxia in renal cell carcinoma (Turcotte *et al.* 2003). Our experiments using DPI, an inhibitor of ROS production, confirm that ROS also participate in the increase in RhoA activity observed in cardiomyocytes subjected to hypoxia.

Recent reports have established some cross-talk between the HIF and RhoA/ROCK pathways. It has been shown that, in some cell types, RhoA is responsible for HIF-1α mRNA and/or protein induction in low oxygen conditions (Turcotte *et al.* 2003; Hayashi *et al.* 2005; Takata *et al.* 2008). In NRVMs, the time courses of the hypoxic regulation of HIF-1α protein and RhoA activity are similar (Bautista *et al.* 2009; this study). Our data show that inhibition of RhoA activity reduces the hypoxic stabilization of HIF-1α, indicating that the small monomeric G protein is involved in HIF-1α regulation in NRVMs. To our knowledge, this is the first study to report HIF-1α regulation by RhoA in cardiac myocytes. As HIF-1α participates in the hypoxic regulation of $Ca_v3.2$ mRNA, and RhoA is involved in HIF-1α regulation, we investigated whether RhoA participates in $Ca_v3.2$ upregulation. Pharmacological inhibition of RhoA or ROCK partly blocked the upregulation of $Ca_v3.2$ mRNA observed in hypoxia. Furthermore, inhibition of ROS production with DPI, which blocked the increase in RhoA activity observed in hypoxia, also blocked the hypoxic upregulation of $Ca_v3.2$ mRNA. These data indicate that the RhoA/ROCK signalling pathway participates in the signalling cascade responsible for $Ca_v3.2$ hypoxic regulation.

In summary, our results demonstrate the hypoxic regulation of T-type Ca^{2+} channels in cardiac myocytes and suggest the existence of cross-talk between RhoA/ROCK and HIF pathways. Our data show that hypoxia induces an increase in RhoA activity and stabilizes HIF-1α protein. Stabilization of HIF-1α mediates the upregulation of $Ca_v3.2$ mRNA and the subsequent increase in T-type Ca^{2+} current observed in NRVMs exposed to hypoxia. Furthermore, we also observed the upregulation of $Ca_v3.2$ mRNA and current in response to hypoxia/DMOG in adult ventricular myocytes. However, our

studies were mainly conducted in NRVMs because they are more suitable for long-term culture, allowing the performance of mechanistic gene regulation studies. Nevertheless, as many of the signalling pathways active in NRVMs are reactivated in pathological conditions in adult cardiomyocytes, our studies could be important in cardiovascular disorders associated with hypoxia. Although further studies will be necessary to determine the functional consequences of this T-type current upregulation in both neonatal and adult cardiac myocytes, an increase in the expression of T-type channels should increase Ca^{2+} influx into the cardiomyocyte cytoplasm, and could contribute to the pathogenesis of diseases associated with Ca^{2+} overload (Ferron *et al.* 2003; Vassort *et al.* 2006; Ono & Iijima, 2010), including cardiac hypertrophy (Lory *et al.* 2006). On the other hand, re-expression of low voltage-activated T-type Ca^{2+} channels in adult ventricular myocytes during cardiac pathologies could confer automaticity to these cells and would represent a pro-arrhythmogenic condition (Vassort *et al.* 2006).

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Additional information

Competing interests

None.

Author contributions

All authors designed the experiments. P.G.-R., M.J.C., D.F.B. and A.C. performed the experiments. P.G.-R., J.U., J.L.-B. and A.C. analysed and interpreted the data and drafted the manuscript. All authors approved the final version for publication.

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