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Chapter XX

T-type Ca^{2+} channel regulation by CO: a mechanism for control of cell proliferation

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Abstract. T-type Ca^{2+} channels regulate proliferation in a number of tissue types, including vascular smooth muscle and various cancers. In such tissues, up-regulation of the inducible enzyme heme oxygenase-1 (HO-1) is often observed. HO-1 degrades heme to generate carbon monoxide (CO) along with Fe^{2+} and biliverdin. Since CO is increasingly recognized as a regulator of ion channels, we have explored the possibility that it may regulate proliferation via modulation of T-type Ca^{2+} channels.

Whole-cell patch-clamp recordings revealed that CO (applied as the dissolved gas or via CORM donors) inhibited all 3 isoforms of T-type Ca^{2+} channels (Cav3.1-3.3) when expressed in HEK293 cells with similar IC_{50} values, and induction of HO-1 expression also suppressed T-type currents. CO / HO-1 induction also suppressed the elevated basal $[Ca^{2+}]_i$ in cells expressing these channels and reduced their proliferative rate to levels seen in non-transfected control cells.

Proliferation of vascular smooth muscle cells (both A7r5 and human saphenous vein cells) was also suppressed either by T-type Ca^{2+} channel inhibitors (mibefradil and NNC 55-0396), HO-1 induction or application of CO. Effects of these blockers and CO were non additive. Although L-type Ca^{2+} channels were also sensitive to CO, they did not influence proliferation. Our data suggest that HO-1 acts to control proliferation via CO modulation of T-type Ca^{2+} channels.

Keywords: heme oxygenase; carbon monoxide; T-type Ca^{2+} channel; smooth muscle; vascular disease; proliferation

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XX.2 Introduction

Heme oxygenases (HO-1 and HO-2) are enzymes that catalyse the degradation of heme, generating biliverdin, Fe^{2+} and carbon monoxide (CO). The enzymes differ in that HO-2 is widely distributed and constitutively active, whereas HO-1 (also known as heat shock protein 32) is induced by numerous forms of cellular stress, including hypoxia (Kim et al. 2006;Ryter et al. 2006). HO-1 is commonly regarded as protective through reduction of levels of heme (which is itself pro-oxidant), and formation of biliverdin which is rapidly converted to the powerful antioxidant, bilirubin.

Induction of HO-1 is associated with pathological cardiovascular conditions including myocardial infarction, hypertension, atherosclerosis and vascular injury. Although diverse, these important diseases share the common feature of increased levels of vascular smooth muscle cell (VSMC) proliferation (Ryter et al. 2006;Chang et al. 2008). Evidence indicates that HO-1 has anti-proliferative effects which are likely due to its ability to generate CO (Otterbein et al. 2003;Durante et al. 2006;Durante 2003): indeed, CO inhalation reduces VSMC proliferation in intimal hyperplasia which often arises as an unwanted consequence of vessel grafting (Otterbein et al. 2003;Ramlawi et al. 2007). Based in part on such findings, CO (in the form of controlled inhalation levels, or CO-releasing molecules (CORMs)), is being explored as a therapeutic approach for cardiovascular disease (Foresti et al. 2008), although it should be noted that the detailed mechanisms underlying its anti-proliferative effects remaining unknown.

Switching of VSMCs from the contractile to proliferative phenotype is a crucial step in the progression of vascular diseases, as well as in development and repair (Owens et al. 2004;Owens 1995;Wamhoff et al. 2006). Associated with this switch is a dramatic change in the expression of a restricted number of ion channels (Cidad et al. 2010), included amongst which is the T-type Ca^{2+} channel (Perez-Reyes 2003). In contractile VSMCs Ca^{2+} influx via L-type Ca^{2+} channels is a major determinant of vascular tone, but in proliferating VSMCs L-type Ca^{2+} channel expression declines substantially whilst expression of T-type Ca^{2+} channels increases (Richard et al. 1992;Kuga et al. 1996). Much evidence indicates that influx of Ca^{2+} via T-type Ca^{2+} channels is required for proliferation and neointima formation following vascular injury (Kuga et al. 1996;Schmitt et al. 1995;Rodman et al. 2005;Lipskaia et al. 2009;Tzeng et al. 2012).

A number of research groups, including our own, have demonstrated that specific ion channels are targets of regulation by CO, and that via ion channel modulation CO exerts many of its important physiological and pathological actions (Williams et al. 2004;Jaggar et al. 2005;Telezhkin et al. 2011;Scragg et al. 2008;Dallas et al. 2012;Peers et al. 2014). In the present study, we have examined whether T-type Ca^{2+} channels represent another target for modulation by CO and, if so, how this might impact on VSMC proliferation.

XX.3 Methods

XX.3.1 Cell culture

Experiments were performed using HEK293 cells (Boycott et al. 2013), the aortic smooth muscle cell line A7r5 (Kimes and Brandt 1976) and human saphenous vein cells (Duckles et al. 2014). HEK293 cells were cultured in minimum essential medium containing Earle's salts and L-glutamine, and supplemented with 10% (v/v) foetal bovine serum (FBS; Biosera, Ringmer UK), 1% (v/v) non-essential amino acids, 1% (v/v) antibiotic/antimycotic, and 0.1% (v/v) gentamicin. HEK293 cells stably expressing T-type Ca^{2+} channels (Cav3.1,

3.2 or 3.3; a kind gift from E. Perez-Reyes; University of Virginia, USA), were cultured in wild type (WT) HEK293 media supplemented with 1mg/ml G-418. Cells were kept in a humidified incubator at 37ºC (95% air: 5% CO_2) and passaged weekly. A7r5 cells (from the European Collection of Cell Cultures, Porton Down UK) were grown in A7r5 complete media, consisting of DMEM containing 10% FBS (Biosera, Ringmer UK) and 1% glutamax (Gibco, Paisley UK). Human saphenous vein smooth muscle cells (HSVSMCs) were isolated from the saphenous vein (SV) of anonymous patients undergoing coronary artery bypass graft surgery as previously described (Duckles et al. 2014). All cells were kept in a humidified incubator at 37ºC (95% air: 5% CO_2).

XX.3.2 Electrophysiology

Ca^{2+} currents were recorded from HEK293 cells using the whole-cell configuration of the patch-clamp technique at room temperature (21-24ºC) as previously described (Boycott et al. 2013). Pipettes (4–6 MΩ) contained (in mM): CsCl (120), $MgCl_2$ (2), EGTA (10), TEA-Cl (20), HEPES (10), Na-ATP (2), pH 7.2 (adjusted with CsOH), and the perfusate was composed of (in mM): NaCl (95), CsCl (5), $MgCl_2$ (0.6), $CaCl_2$ (15), TEA-Cl (20), HEPES (5), D-glucose (10), pH 7.4 (adjusted with NaOH). Cells were voltage-clamped at -80mV and either repeatedly depolarized to -20mV (200ms, 0.1Hz) or to a series of test potentials ranging from -100mV to +60mV. All currents were low-pass filtered at 2kHz and digitised at 10kHz.

XX3.3 Proliferation Assay

Cells were plated in 24-well plates in complete media ($1x10^4$ cells per well). HSVSMCs were allowed to adhere overnight and subjected to serum free media (SFM) for 2.5 days respectively. A7r5 and HEK293 cells were allowed to adhere for 6 hours and then exposed to SFM overnight. On day 0 of the assay, SFM was removed and 1ml of the relevant complete media added to each well, in addition to any drugs being investigated. To count cells, media was removed, cells were washed with 1ml of Dulbecco's phosphate buffered saline (PBS) and 200µl of 0.05% trypsin-EDTA (Gibco, Paisley UK) was added (pre-warmed to 37^0C). Post-incubation, 800µl of complete media was added and the cell suspension centrifuged (600g for 6 minutes). Following removal of 950µl of media, 50µl of supernatant remained with the cell pellet, which was then re-suspended with 50µl of 0.4% Trypan Blue (Thermo Scientific, Rockford USA) to exclude non-viable cells. Media was retained from one well of each treatment, processed in the same manner as the cell samples, and any cells present were counted as an additional quantification of non-viable cells. Day 0 counts and media counts were performed using a hemocytometer. All other counts were performed using a TC10 Automated Cell Counter (Bio-Rad, Hemel Hempstead UK).

XX3.4 Western Blotting

Cells were grown to 80% confluence in 6-well plates. Wells were replenished with 0.4% serum-containing media plus the required drugs. Post-treatment, the cells were washed with PBS and lysed with Mammalian Protein Extraction Reagent (M-PER™; Thermo Scientific, Rockford USA) containing Complete Mini protease inhibitors (Roche Diagnostics Ltd, Lewes UK). Protein levels were determined using a BCA protein assay kit (Thermo Scientific, Rockford USA). Next, 10-20µg protein in 2x sample buffer (250 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 1% bromophenol blue, and 10% β-mercaptoethanol) was loaded onto 12.5% polyacrylamide gels and separated for ~1 hour at 35mA before being transferred onto 0.2µm polyvinyl difluoride membranes at 30V overnight.

Membranes were blocked using 5% (w/v) non-fat dried milk powder in Tris buffered saline (TBS)-Tween (0.05%) for 1 hour, then incubated with rabbit anti-HO-1 antibody (1:200; SC-10789; Santa Cruz, Dallas USA) for 3 h at 21-24^{o}C. Mouse anti-β-actin raised against the N-terminal of β-actin (1:4000; Sigma, Gillingham UK) was used as a loading control. Membranes were then washed in TBS-Tween (0.05%) and incubated with the corresponding peroxidase-conjugated secondary antibody (1:2000; GE Healthcare, Amersham UK) for 1 hour at 21-24^{o}C. Bands were detected using the enhanced chemi-luminescent method on hyperfilm. Densitometric analysis used Image J (NIH, UK).

XX3.5 Ca^{2+} Microfluorimetry

Cells were plated on circular glass coverslips and allowed to adhere overnight. Cells were washed and incubated with 4μM Fura 2-AM (Invitrogen, Cambridge UK) in HEPES-buffered saline (HBS) for 40 min 21-24^{0}C. HBS contained (in mM): NaCl (135), KCl (5), $MgSO_4$ (1.2), $CaCl_2$ (2.5), HEPES (5), glucose (10), osmolarity adjusted to 300 mOsm with sucrose, and pH adjusted to 7.4. The Fura 2-containing HBS was washed off for 15 min to allow de-esterification. Coverslip fragments were placed in a perfusion chamber on an inverted epifluorescence microscope and cells superfused via gravity at 2-3ml/min. $[Ca^{2+}]_i$ was indicated by fluorescence emission measured at 510nm as a result of alternating excitation at 340nm and 380nm using a Cairn Research ME-SE Photometry system (Cairn Research, Cambridge UK).

Statistical comparisons were made using, as appropriate, paired or unpaired student's T-tests, one way ANOVA with a multiple comparison test, or repeated measures one way ANOVA with a multiple comparison test.

XX.4 Results

XX4.1 CO inhibits T-type Ca^{2+} channels

Whole-cell patch-clamp recordings from HEK293 cells expressing each of the major forms of T-type Ca^{2+} channels (Cav3.1, 3.2 or 3.3) revealed that CO, applied as the CO donor CORM-2, inhibited all 3 isoforms (Fig. XX.1A). These effects were attributable to release of CO, since the inactive form of CORM-2 was without significant effect (not shown). Indeed, all three isoforms displayed similar sensitivity to CORM-2 (IC_{50} ca 3μM in each case). The time course of inhibition (Fig. XX.1B) shows that CO inhibition of currents recorded in Cav3.2-expressing HEK293 cells was irreversible. However, current amplitude recover was observed following exposure to the reducing agent dithiothreitol (DTT; 1mM), as exemplified in Fig XX1B (lower plot). The ability of DTT to reverse CO inhibition was exploited to examine whether HO-1 induction led to tonic inhibition of T-type currents: exposure of cells to hemin (iron protoporphyrin IX (200μM)) caused a time-dependent induction of HO-1, as determined by western blot (Fig. XX.1C). Following HO-1 induction, the ability of DTT to augment currents was dramatically enhanced (Fig. XX.1C), consistent with the idea that HO-1 induction led to increased CO levels, thereby causing tonic inhibition of Cav3.2-mediated T-type currents.

XX4.2 CO modulation of $[Ca^{2+}]_i$ and proliferation in Cav3.2 expressing cells

To investigate the consequences of CO inhibition of T-type Ca^{2+} channels on cell function, we next explored the ability of CO to regulate $[Ca^{2+}]_i$ and cellular proliferation. Experiments were restricted to studying the effects of CO in Cav3.2-expressing cells. As

exemplified in Fig. XX.2A, basal $[Ca^{2+}]_i$ was significantly elevated in cells expressing this channel, as

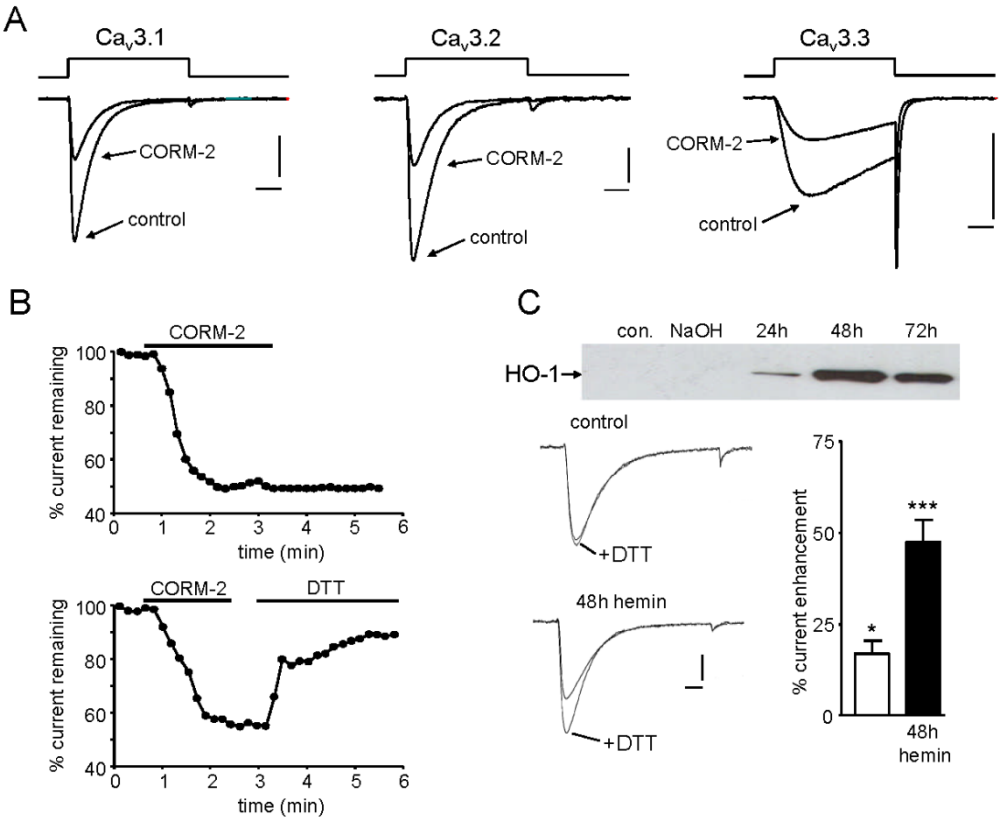

Fig. XX.1. CO inhibits T-type channels. (A) currents evoked in a HEK293 cell stably expressing Cav3.1 (left) Cav3.2 (middle) or Cav3.3 (right) before and during exposure to CORM-2 (3μM) as indicated. Currents were evoked by step depolarizations from -90mV to -30mV. Scale bars in each case: vertical, 0.5nA, horizontal, 20ms. (B) Upper; example time-series plot illustrating normalized current amplitudes evoked by step depolarizations from -90mV to -30mV in a Cav3.2-expressing cell. For the period indicated by the bar, CORM-2 (3μM) was applied via the perfusate. Lower; as upper, except that, following washout of CORM-2, the cell was exposed to dithiothreitol (DTT, 1mM) for the period indicated by the second bar. (C) Upper; western blot illustrating the time-dependent induction of HO-1 following exposure to hemin (200μM). Lower, example currents showing the effects of DTT (1mM) on untreated cells (control) or those in which HO-1 had been induced following exposure to hemin (200μM, 48h) . Scale bars: 0.1nA, 10ms. Right; plot of the mean (± s.e.m.) % enhancement of currents in control (n=9) and hemin-treated cell caused by DTT (n=9). Data obtained from currents evoked by step depolarizations from -90mV to -30mV. Statistical significance: * P<0.05; *** P<0.001 as compared with controls. Adapted from (Boycott et al. 2013;Duckles et al. 2014) with permission.

compared with wild type (WT; untransfected) cells, and removal of extracellular Ca^{2+} (replaced with 1mM EGTA) caused a much greater fall of $[Ca^{2+}]_i$ as compared with WT cells, consistent with the idea that the elevated basal $[Ca^{2+}]_i$ was attributable to tonic Ca^{2+} influx via T-type Ca^{2+} channels. Induction of HO-1 (using cobalt protoporphyrin, CoPPIX) also reduced basal $[Ca^{2+}]_i$ (Fig. xx.2B), as did exposure to CO using the water soluble CO donor, CORM-3. This donor was without effect in WT cells (Fig. XX.2C). Proliferation was also monitored in these same cells. As shown in Fig. XX.2D, expression of Cav3.2 led to a dramatic increase in proliferation as compared with WT cells. This increase was presumably due to tonic Ca^{2+} influx via Cav3.2 channels, since proliferation was reduced by the T-type Ca^{2+} channel inhibitor mibefradil, and also by CORM-3. Note that (i) these agents were non-additive, (ii) that either mibefradil or CORM-3 reduced proliferation rates in Cav3.2-expressing cells to levels observed in WT cells, and (iii) these agents were without effect on

proliferation in WT cells, suggesting that increased proliferation was entirely attributable to T-type Ca^{2+} channel activity, which in turn can be regulated by CO.

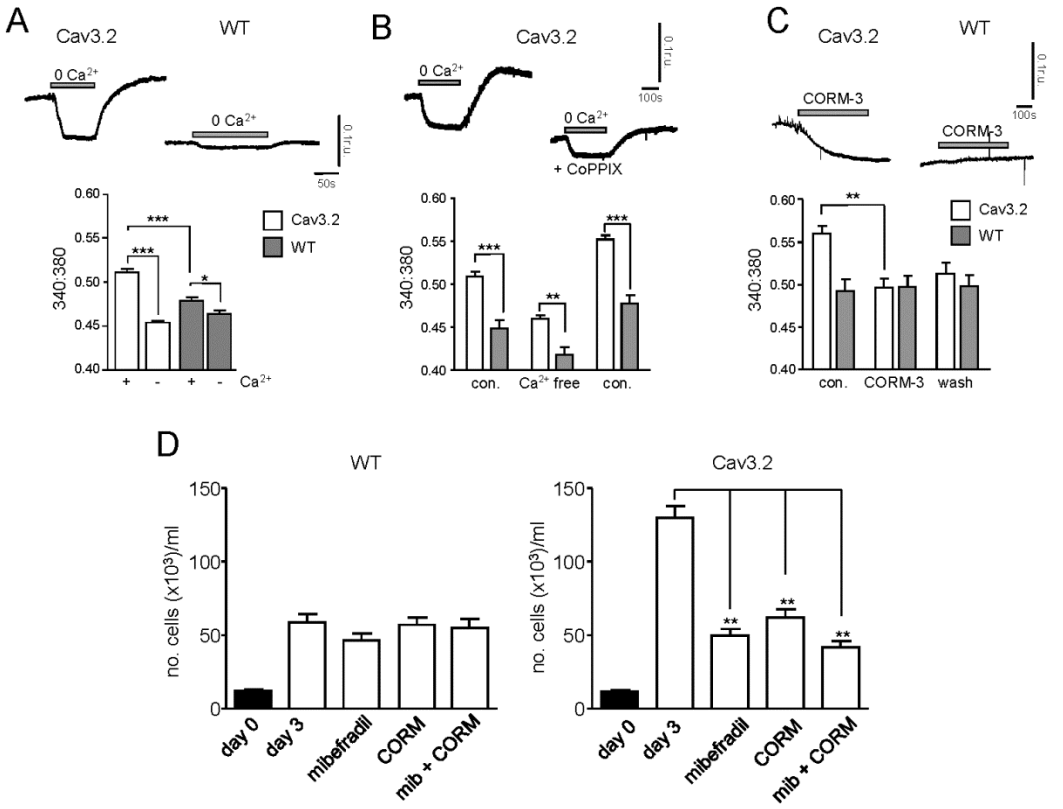

Fig. XX.2. CO modulates $[Ca^{2+}]_i$ and proliferation in Cav3.2 expressing cells. (A) Upper traces show examples of basal $[Ca^{2+}]_i$ recorded in Cav3.2-expressing and WT HEK293 cells. For the periods indicated by the horizontal bars, extracellular Ca^{2+} was replaced with 1mM EGTA. Below; Bar graph illustrating the mean basal $[Ca^{2+}]_i$ levels (with s.e.m. bars) recorded in Cav3.2-expressing cells (open bars, n=6) and WT cells (shaded bars, n=6) in the presence and absence of extracellular Ca^{2+}, as indicated. (B) Upper traces; examples of basal $[Ca^{2+}]_i$ recorded in Cav3.2-expressing cells either with no pre-treatment, or exposed to 10µM CoPPIX for 48h to induce HO-1 expression (+CoPPIX). For the periods indicated by the horizontal bars, extracellular Ca^{2+} was replaced with 1mM EGTA. Below; Bar graphs illustrating the mean (± s.e.m.) basal $[Ca^{2+}]_i$ levels recorded in Cav3.2-expressing cells (n=16) before (con.), during (Ca^{2+} free) and after (con.) removal of extracellular Ca^{2+}. Statistical significance: ** P<0.01, *** P<0.001 (C) Upper traces show examples of basal $[Ca^{2+}]_i$ recorded in Cav3.2-expressing and WT HEK293 cells and the effects of CORM-3 (3µM) applied for the periods indicated by the horizontal bars. Below; Bar graph illustrating the mean (± s.e.m.) basal $[Ca^{2+}]_i$ levels recorded in Cav3.2-expressing cells (open bars, n=5) and WT cells (shaded bars, n=6) before (con.), during (CORM-3) and after (wash) exposure to CORM-3. Statistical significance: * P<0.05; ** P<0.01, *** P<0.001 as compared with appropriate controls. Data analysed via paired or unpaired t-test as appropriate. (D) Bar graphs illustrating the effects of mibefradil and CORM-3 (applied separately or together, as indicated) on proliferation measured on day 3 in WT (left) and Cav3.2-expressing HEK293 cells (right). Each bar represents mean (± s.e.m.) proliferation determined from 9 repeats. Statistical significance: ** P<0.01 as compared with controls. Data analysed via repeated ratio measures one way ANOVA followed by Dunnett's multiple comparison test. Taken from (Duckles et al. 2014) with permission.

XX4.3 CO modulates proliferation in VSMCs

To examine whether CO could also modulate proliferation via modulation of native T-type Ca^{2+} channels in VSMCs, we examined its effects in both A7r5 cells and HSVSMCs. As shown in Fig. XX.3A, although A7r5 cells express both L- and T-type Ca^{2+} channels

(Duckles et al. 2014), proliferation was only reduced by mibefradil and not nifedipine, indicating that T-type but not L-type channels regulated proliferation in these cells.

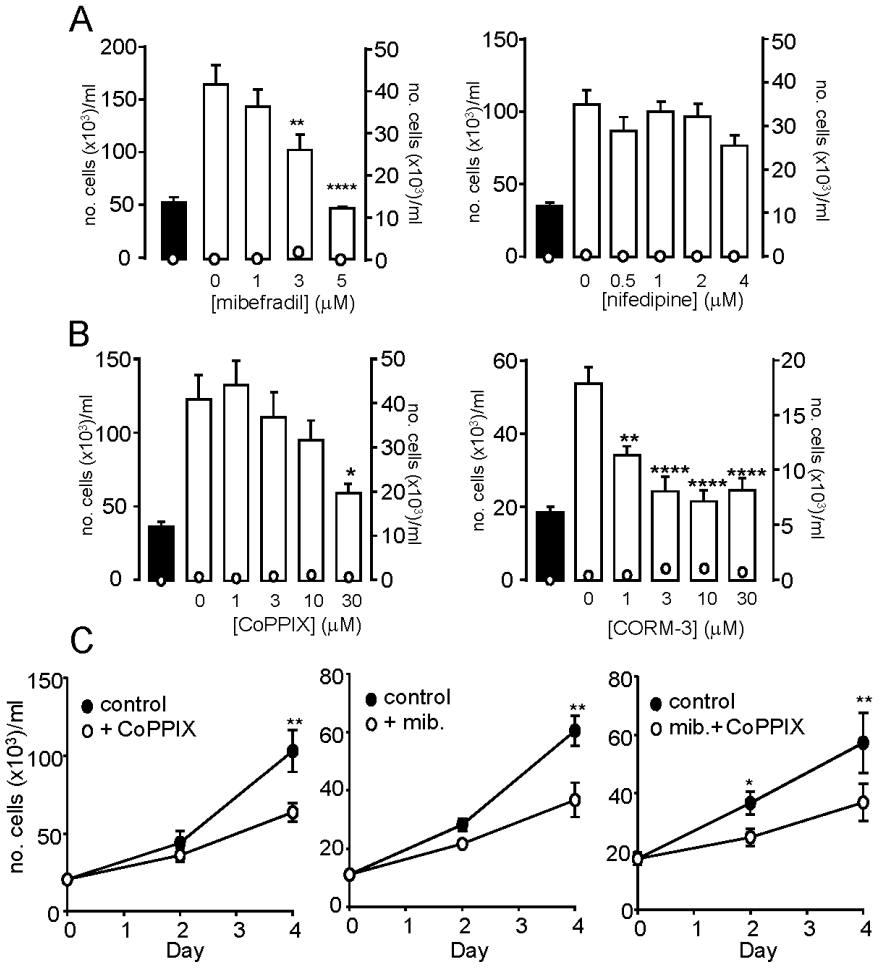

Fig. XX.3. T-type Ca^{2+} channel inhibitors suppress proliferation of A7r5 cells and HSVSMCs. (A) Bar graphs showing the proliferative response (mean ± s.e.m) of A7r5 cells to increasing concentrations of specified drugs. Proliferation (plotted as bar graphs, corresponding to the left-hand Y axis) was monitored on Day 0 (solid bars) and on Day 3 (open bars) in the absence or presence of mibefradil (n=4) or nifedipine (n=3). (B) as (A), except cells were exposed to CoPPIX to induce HO-1, or to CORM-3, as indicated. The open circles show the corresponding non-viable cell count (plotted against corresponding right-hand Y-axis). Statistical significance: * $p<0.05$, ** $p<0.01$, **** $p<0.0001$ vs Day 3 Control (no drug). Data analysed via repeated ratio measures one way ANOVA followed by Dunnett's multiple comparison test. (C) Line graphs showing proliferation of HSVSMCs monitored over a 4-day period, in the absence of drug treatment (solid circles), or during HO-1 induction with 3μM CoPPIX (open symbols, left), or in the presence of 3μM mibefradil (open circles, rmiddle), or during simultaneous application of 3μM mibefradil and 3μM CoPPIX (open circles, right). Each point represents mean ± s.e.m. (n=5). Statistical significance: * $p<0.05$, ** $p<0.01$. Data analysed via repeated measures one way ANOVA followed by Sidak's multiple comparison test between control and treated groups for each timepoint. Taken from (Duckles et al. 2014) with permission.

Consistent with their effects in Cav3.2-expressing HEK293 cells, HO-1 induction or CO exposure (using CORM-3) also significantly reduced proliferation (Fig. XX.3B). Similarly, in HSVSMCs both HO-1 induction and mibefradil separately reduced proliferation, and combining both maneuvers did not suppress proliferation further (Fig. XX.3C). These data are consistent with data obtained in HEK293 cells indicating that VSMC proliferation is also regulated by T-type Ca^{2+} channel activity, and that CO inhibition of T-type channels could significantly suppress proliferation.

XX.5 Discussion

The switch of VSMCs from a contractile to a proliferative phenotype is a complex process which is, in part, regulated by Ca^{2+} influx (Barbado et al. 2009;Owens et al. 2004).

Various routes of Ca^{2+} entry into VSMCs have been proposed as important for proliferation, including TRPC channels (Kumar et al. 2006) and the STIM1 / Orai pathway (Zhang et al. 2011). There is also a substantial body of evidence implicating T-type Ca^{2+} channels in the process of proliferation and neointima formation following vascular injury (Kuga et al. 1996;Schmitt et al. 1995). In more recent times, Cav3.1 has been identified as the key T-type Ca^{2+} channel required for VSMC proliferation (Tzeng et al. 2012). Our data in A7r5 and HSVSMCs are consistent with an important role for native T-type Ca^{2+} channels in proliferation. Thus, in A7r5 cells, despite the presence of L-type Ca^{2+} channels (Duckles et al. 2014), proliferation appeared selectively regulated by T-type Ca^{2+} channels, and this was also the case in HSVSMCs (Fig. XX.3). The observation that proliferation was sensitive to mibefradil implies that tonic Ca^{2+} influx via T-type Ca^{2+} channels occurs in these cells. We present no direct evidence of this in VSMCs, but in HEK293 cells expressing Cav3.2, tonic Ca^{2+} influx via these channels is indeed evident (Fig. XX.2). This presumably arises from the ability of these channels to generate a window current (Perez-Reyes 2003), which arises from a small proportion of the T-type Ca^{2+} channel population being partially activated and not fully inactivated at the cell's resting membrane potential.

Our findings indicate that proliferation can be suppressed by induction of HO-1 in VSMCs, and that this occurs via formation of CO by HO-1. Thus, CO was able to mimic the suppressive effects of HO-1 induction in VSMCs (Fig. XX.3) and, in HEK293 cells expressing Cav3.2, HO-1 induction or CO reduced basal $[Ca^{2+}]_i$ and suppressed proliferation. These findings are consistent with numerous studies indicating that HO-1 induction suppresses proliferation in vascular diseases (Cao et al. 2009;Araujo et al. 2012). Furthermore, other studies implicate CO as the means by which HO-1 suppresses proliferation (Otterbein et al. 2003;Ramlawi et al. 2007). In light of the direct evidence from recombinant studies (Fig. XX.1), we propose that HO-1 regulation of VSMC proliferation is attributable to the ability of CO to inhibit T-type Ca^{2+} channels. Thus, CO inhibits all three isoforms of T-type channel, and inhibition is observed when CO is applied directly, or when HO-1 is induced (Fig. XX.1). These results, coupled with the observations that CO or HO-1 induction suppresses T-type Ca^{2+} channel-dependent proliferation in VSMCs (Fig. XX.3) imply that the ability of CO to inhibit T-type Ca^{2+} channels is important in VSMC. This implication is further supported by the observation that the effects of HO-1 induction and mibefradil exposure on proliferation of HSVSMCs were not additive.

In summary, our data provide a novel signalling pathway to account for the anti-proliferative effects of HO-1 observed in vascular diseases. This pathway has the potential for targeting in future strategies for the treatment of vascular diseases. There are also important implications arising from our studies for other diseases including various cancers where T-type Ca^{2+} channels have been shown to regulate proliferation (Panner and Wurster 2006;Heo et al. 2008).

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