Inhibition of L-Type Ca^{2+} Channels by Carbon Monoxide

M.L. Dallas, J.L. Scragg and C. Peers

Abstract Inhibition of K^+ channels in glomus cells underlies excitation of the carotid body by hypoxia. It has recently been proposed that hypoxic inhibition involves either activation of AMP activated protein kinase (AMPK) or inhibition of carbon monoxide (CO) production by heme oxygenase 2 (HO-2). In the vasculature, L-type Ca^{2+} channels are also O_2 sensitive. Here, we have investigated the possible involvement of either AMPK or CO in the hypoxic inhibition of L-type Ca^{2+} channels. Using whole-cell patch clamp recordings from HEK293 cells stably expressing the human cardiac α_{1C} Ca^{2+} channel subunit, we found that pre-treatment of cells with AICAR (to activate AMPK) was without effect on Ca^{2+} currents. CO, applied via the donor molecule CORM-2 caused reversible, voltage-independent Ca^{2+} channel inhibition of up to *ca.* 50%, whereas its inactive form (iCORM) was without significant effect. Effects of CO were prevented by the antioxidant MnTMPyP, but not by inhibition of NADPH oxidase (with either apocynin or diphenyleneiodonium), or xanthine oxidase (with allopurinol). Instead, inhibitors of complex III of the mitochondrial electron transport chain and a mitochondrial-targeted antioxidant (Mito Q), prevented the effects of CO. Our data suggest that hypoxic inhibition of L-type Ca^{2+} channels does not involve AMPK or CO. However, the known cardioprotective effects of HO-1 could arise from an inhibitory action of CO on L-type Ca^{2+} channels.

Keywords Ca^{2+} channel · L-type · Carbon monoxide · Reactive oxygen species · Mitochondria · Splice variant · Patch clamp · AMP kinase · Electron transport chain

1 Introduction

The membrane hypothesis for chemotransduction supports the concept that hypoxia (and other chemostimuli) excite the carotid body by inhibiting K^+ channels in glomus cells (Kumar, 2007). This leads to their depolarization, voltage-gated Ca^{2+}

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entry and hence release of neurotransmitters onto afferent chemosensory fibres. The principal mechanisms underlying hypoxic inhibition of K^+ channels remains to be fully elucidated. However, two proposed mechanisms have received attention in recent years, both involving enzymes coupled to channel activity. Firstly, heme oxygenase-2 (HO-2) was found to co-localise with O_2 sensitive maxiK channels in a recombinant expression system. In the presence of O_2 and NADPH, HO-2 produces CO during the breakdown of heme. CO increases maxiK channel activity, and so during hypoxia, CO levels decline thereby reducing K^+ channel activity (Williams et al. 2004). This mechanism was confirmed in rat glomus cells, though may not be so prominent in other species (Ortega-Saenz et al. 2006). Secondly, the "metabolic fuel gauge" enzyme AMP activated protein kinase (AMPK (Hardie et al. 2006)) was proposed to be activated (possibly by a shift in the AMP:ATP ratio) in glomus cells during hypoxia (Evans et al. 2005). This enzyme was subsequently demonstrated to phosphorylate maxiK channels directly (Wyatt et al. 2007) and, in so doing, suppress their activity. Furthermore, AMPK activation caused glomus cell depolarization, raised $[Ca^{2+}]_i$ and excited afferent chemosensory fibres (Wyatt et al. 2007) and inhibition of AMPK activity suppressed hypoxic excitation of the carotid body.

Hypoxia also suppresses the activity of L-type Ca^{2+} channels in the vasculature (Franco-Obregon et al. 1995), an effect which may contribute to systemic hypoxic vasodilation. However, in contrast to the studies described above for hypoxic inhibition of maxiK channels, no mechanisms have yet been proposed to account for this action of hypoxia on Ca^{2+} channels. The present study was therefore designed to investigate whether hypoxic inhibition of recombinant L-type Ca^{2+} channels – which appears indistinguishable from effects seen on native channels (Fearon et al. 1997) – involves either activation of AMPK or altered CO production.

2 Methods

HEK293 cells stably transfected with the hHT splice variant of the human cardiac L-type Ca^{2+} channel α_{1C} subunit (Fearon et al. 2000) were cultured in growth medium comprising MEM with Earle's salts and L-glutamine, supplemented with 9% (v/v) fetal calf serum (Globepharm, Esher, Surrey, UK), 1% (v/v) non-essential amino acids, 50 μg/ml gentamicin, 100 units/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin in a humidified atmosphere of air/CO_2 (19:1) at 37°C. All culture reagents were purchased from Gibco-BRL (Paisley, UK) unless otherwise stated.

HEK293 cells attached to coverslip fragments were placed in a perfused (2–4 ml/min) chamber and whole-cell patch-clamp recordings were made as previously described (Fearon et al. 2000). Perfusate contained (in mM): NaCl, 95; CsCl, 5; $MgCl_2$, 0.6; $BaCl_2$, 20; HEPES, 5; D-glucose, 10; TEA-Cl 20 (pH 7.4, 21–24°C). Patch electrodes (resistance 4–7 MΩ) contained (in mM): CsCl, 120; TEA-Cl, 20; $MgCl_2$, 2; EGTA, 10; HEPES, 10; ATP, 2 (pH adjusted to 7.2 with CsOH). Cells were clamped at –80 mV and whole cell capacitance determined from

analogue compensation. Series resistance compensation of 70–90% was applied. Whole-cell currents were evoked by 100 ms step depolarizations to various test potentials (0.1 Hz) and leak-subtraction was applied as previously described (Fearon et al. 2000). Evoked currents were filtered at 1 kHz, digitized at 2 kHz and current amplitudes measured over the last 10–15 ms of each step depolarization since Ba^{2+} was used as the charge carrier, as they displayed little or no inactivation during step depolarizations. Currents showing notable run-down before CORM-2 application were discarded.

All voltage-clamp and analysis protocols were performed with the use of an Axopatch 200A amplifier/Digidata 1200 interface controlled by Clampex 9.0 software (Molecular Devices, Foster City, CA). Offline analysis was performed using the data analysis package Clampfit 9.0 (Molecular Devices, Foster City, CA). Results are presented as means ± S.E.M., and statistical analysis performed using unpaired Student's *t*-tests where $P < 0.05$ was considered statistically significant.

3 Results

3.1 AMPK Activation Does Not Alter Ca^{2+} Channels

To investigate the possible involvement of AMPK in the hypoxic modulation of Ca^{2+} channels, HEK293 cells were exposed to AICAR (1 mM) for at least 20 min at 37°C. AICAR is metabolised intracellularly to generate an AMP-like molecule, thereby activating AMPK (Evans et al. 2005). This approach has previously been employed to demonstrate AMPK regulation of maxiK channels (Wyatt et al. 2007). As shown in Fig. 1, this approach was completely without effect on Ca^{2+} channel currents throughout the range of activating test potentials studied. Thus, it appears unlikely that AMPK activation can account for hypoxic inhibition of L-type Ca^{2+} channels.

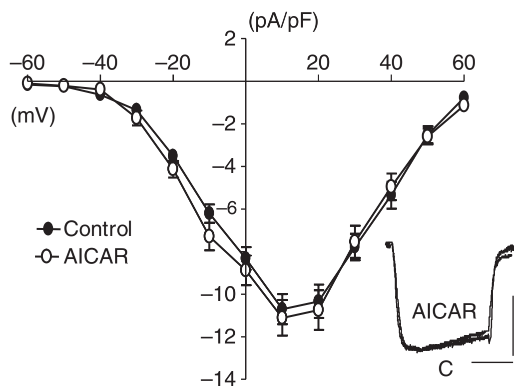

Fig. 1 Mean (with S.E.M. bars, n=8) current-density versus voltage relationships measured in HEK293 cells expressing α_{1C} subunits. Cells were either untreated, or exposed to AICAR (1 mM) for at least 20 min prior to recordings. Inset, example currents from the two cells groups. Currents evoked by step depolarizations from −80 mV to +10 mV. Scales bars; 100pA *vertical*, 50 ms *horizontal*

3.2 CO Inhibits Ca^{2+} Channels

Exposure of HEK293 cells to the established CO donor CORM-2 caused marked inhibition of currents, as exemplified in Fig. 2A. Inhibition was slowly reversible and not associated with changes in current kinetics (Fig. 2A). The effects of CORM-2 were concentration-dependent, with an IC_{50} of ca. 15 μM. Importantly, currents were largely unaffected (∼10% inhibition) either by the vehicle, DMSO, or by the inactive breakdown product, iCORM-2 (Fig. 2B) indicating that the effects of CORM-2 were largely due to its release of CO, as has previously been established (Williams et al. 2004; Boczkowski et al. 2006; Chatterjee, 2007). In addition,

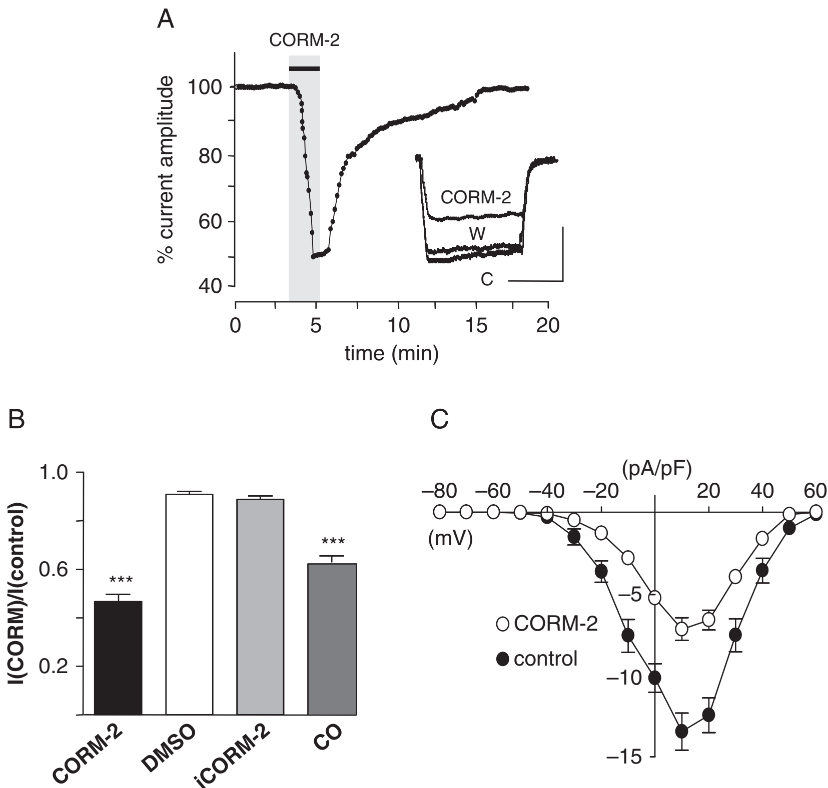

Fig. 2 (**A**) Time course of the inhibitory effect of CO on Ca^{2+} currents in HEK293 cells expressing α_{1C} subunits. CORM-2 (30 μM) was applied for period indicated by bar. Each point is the current amplitude evoked by a depolarization from −80 to +10 mV (100 ms, 0.1 Hz). Inset, superimposed example currents (C; control, W; wash). Scale bars; 100pA vertical, 50 ms horizontal. (**B**) Bar graph showing mean (± s.e.m.) inhibitory effects of CORM-2, vehicle (DMSO), the inactive molecule (iCORM-2) and CO itself. ***$P < 0.001$ vs. the response to iCORM-2. (**C**) Mean (with s.e.m. bars, n=10) current-density versus voltage relationships measured in HEK293 cells expressing α_{1C} subunits. Cells were either untreated (*closed circles*), or exposed to 30 μM CORM-2 (*open circles*)

we exposed cells to dissolved CO and this caused a similar degree of reversible inhibition (Fig. 2B). Full current-voltage relationships indicated that the effects of CO were apparent at all activating test potentials (Fig. 2C), indicating a lack of voltage-dependence.

3.3 CO Increases Mitochondrial ROS Production

CO can modulate a variety of signalling pathways to exert a diverse array of cellular effects (Wu and Wang 2005; Kim et al. 2006). We therefore took a pharmacological approach to investigate candidate mechanisms underlying the ability of CO to inhibit L-type Ca^{2+} channels. Results are summarized in Table 1.

In sum, we found that the inhibitory effects of CO were fully prevented by either the antioxidant MnTMPyP (100 μM) or by the reducing agent, dithiothreitol (DTT; 2 mM). Thus, CO modulation of Ca^{2+} currents appeared to involve reactive oxygen species (ROS). To identify the source of ROS, we inhibited xanthine oxidase with allopurinol (1 μM) and NADPH oxidase with apocynin (30 μM) and diphenyleneiodonium (3 μM). In the presence of these drugs, CO still inhibited Ca^{2+} currents significantly. Next, we examined mitochondria as the source of ROS and found that the mitochondria-targeted antioxidant, MitoQ (250 nM) prevented the actions of CO. Furthermore, both antimycin A (3 μM) and stigmatellin (1 μM), but not rotenone (2 μM), prevented the actions of CO, indicating that CO exerted its effects via ROS production specifically from complex III of the electron transport chain.

4 Discussion

The present work clearly indicates that hypoxia is unlikely to inhibit L-type Ca^{2+} channels either via AMPK activation or via inhibition of HO-2, as has been proposed for hypoxic modulation of maxiK channels (see Introduction). AMPK activation

Table 1 Pharmacological manipulation of the inhibition of L-type Ca^{2+} channels by CO (applied as the CO donor, CORM-2, at 30 μM). Inhibition is expressed as percentage ± S.E.M. taken from at least 6 recordings. Significance compared with CORM-2 vehicle alone

Compound	Inhibition of Ca^{2+} current (%)	Significance
None	53.2 ± 2.3	$P < 0.001$
MnTMPyP	14.5 ± 3.4	n.s.
DTT	10.7 ± 1.1	n.s.
Allopurinol	52.3 ± 0.2	$P < 0.001$
Diphenyleneiodonium	41.6 ± 4.2	$P < 0.001$
Apocynin	37.8 ± 2.1	$P < 0.001$
MitoQ	10.8 ± 3.6	n.s.
Rotenone	46.1 ± 3.0	$P < 0.001$
Antimycin A	13.3 ± 2.7	n.s.
Stigmatellin	15.3 ± 4.3	n.s.

was without effect on Ca^{2+} currents, and the HO-2 product, CO *inhibited* currents, whereas enhancement would be expected if it were to account for hypoxic inhibition. Thus, a novel, as yet undetermined mechanism must account for this action of hypoxia on Ca^{2+} channels. Nevertheless, our study has revealed a novel inhibitory effect of CO which occurs via an increase in ROS production specifically from mitochondria. Cellular ROS production can occur at numerous sites, including NADPH oxidases and xanthine oxidase, and CO can modulate NADPH oxidase (Taille et al. 2005). However, we show that mitochondria are the source of ROS increases evoked by CO. Thus, both a general antioxidant (MnTMPyP) and a mitochondrial targeted one (MitoQ), but not inhibition of NADPH or xanthine oxidases, prevented the actions of CO (Table 1). This is in accordance with previous studies (Taille et al. 2005; Zuckerbraun et al. 2007; D'Amico et al. 2006). CO binds to complex IV (cytochrome c oxidase) of the electron transport chain (see D'Amico et al. 2006), thereby presumably inhibiting its acceptance of electrons from complex III and so causing more to leak and form ROS. Indeed, the effect of CO was prevented by complex III (but not complex I) inhibition (Table 1).

Although these studies have not identified the mechanism of hypoxic inhibition of Ca^{2+} channels, the inhibition by CO is likely to be of physiological significance. Cardiac atrial and ventricular myocytes express HO-1 and HO-2 which both generate CO (together with biliverdin and Fe^{2+} by heme catabolism) and HO-1 levels can be increased by various stress factors (Ewing et al. 1994) including myocardial infarction (Lakkisto et al. 2002) and chronic exposure to hipoxia (Grilli et al., 2003). CO limits the cellular damage of ischemia/reperfusion injury in the heart (Clark et al. 2003). Indeed, greater cardiac damage is seen following ischemia/reperfusion injury in HO-1 knockout mice (Yet et al. 1999). Conversely, HO-1 over-expression in the heart reduces infarct size and other markers of damage following ischemia/reperfusion injury (Yet et al. 2001). CO also improves cardiac blood supply through coronary vessel dilation and reduces cardiac contractility (McGrath 1984). Our data provide a mechanism to account for these protective effects of CO, through inhibition of L-type Ca^{2+} channels via mitochondrial ROS production.

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