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REVIEW

Diverse mechanisms underlying the regulation of ion channels by carbon monoxide

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Carbon monoxide (CO) is firmly established as an important, physiological signalling molecule as well as a potent toxin. Through its ability to bind metal-containing proteins, it is known to interfere with a number of intracellular signalling pathways, and such actions can account for its physiological and pathological effects. In particular, CO can modulate the intracellular production of reactive oxygen species, NO and cGMP levels, as well as regulate MAPK signalling. In this review, we consider ion channels as more recently discovered effectors of CO signalling. CO is now known to regulate a growing number of different ion channel types, and detailed studies of the underlying mechanisms of action are revealing unexpected findings. For example, there are clear areas of contention surrounding its ability to increase the activity of high conductance, Ca^{2+}-sensitive K^+ channels. More recent studies have revealed the ability of CO to inhibit T-type Ca^{2+} channels and have unveiled a novel signalling pathway underlying tonic regulation of this channel. It is clear that the investigation of ion channels as effectors of CO signalling is in its infancy, and much more work is required to fully understand both the physiological and the toxic actions of this gas. Only then can its emerging use as a therapeutic tool be fully and safely exploited.

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Abbreviations
CORM, CO-releasing molecule; eNOS, endothelial NOS; HO-1(2), haem oxygenase-1 (-2); I/R, ischaemia/reperfusion; LQT-3, long QT-3; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; Trx-1, thioredoxin

Introduction

The public perception of carbon monoxide (CO) is that of a dangerous toxin, and with good reason: this colourless and odourless gas accounts for the majority of fatalities arising from accidental poisoning (Meredith and Vale, 1988; Cobb and Etzel, 1991; Varon *et al.*, 1999). It is primarily generated by the partial oxidation (usually occurring via incomplete combustion) of hydrocarbon sources and is a significant component of vehicle exhaust fumes, tobacco smoke, and gas or wood-burning appliances (Soslow and Woolf, 1992). Acute toxicity arises primarily from tissue hypoxia, a consequence of the high-affinity binding of CO to haemoglobin, which prevents oxygen transport and delivery to tissues (Kolarzyk, 1994). However, as discussed below, this does not account for all of the toxic actions of this gas: more insidious are the

effects of sub-lethal, prolonged CO exposure (Meredith and Vale, 1988; Prockop and Chichkova, 2007), which represent a far greater danger to the public, particularly the elderly population; symptoms are difficult for patients to recognize, and can also be difficult to diagnose when medical advice is sought (Harper and Croft-Baker, 2004).

Given this bleak picture of CO toxicity, combined with public awareness campaigns to promote proper maintenance of household heaters, boilers, etc. (e.g. http://www.carbonmonoxide.ie/htm/week.htm), it seems counter-intuitive to consider CO as a beneficial, physiologically important molecule, yet within the scientific and medical research communities, this is now a well-established fact. The progress made in our understanding of the biology of CO has developed rapidly and has provided opportunities for development of new therapeutic strategies for the treatment of numerous clinical conditions (Foresti *et al.*, 2008; Motterlini and Otterbein, 2010). This review will discuss briefly both the deleterious and beneficial effects of CO exposure, and how such effects involve specific intracellular signalling pathways. Most specifically, we describe how ion channels are emerging as important effector target molecules for many of the effects of CO.

Deleterious effects of CO

Given the disruption to oxygen transport caused by CO inhalation, it is perhaps not surprising that the major organs most sensitive to CO-induced damage are those that normally consume most oxygen; the heart and brain. However, damage to these and other tissues can also reflect additional actions of CO. In fact, many features of CO toxicity are not observed following damage induced under hypoxic or ischaemic conditions (Stoller, 2007), and often do not correlate well with carboxyhaemoglobin levels (Carnevali *et al.*, 1987; Gandini *et al.*, 2001). Such additional actions of CO, as discussed later and shown schematically in Figure 1, include its ability to stimulate mitochondrial reactive oxygen species (ROS) generation (Zuckerbraun *et al.*, 2007; Bilban *et al.*, 2008; Piantadosi, 2008), which may reflect a form of 'oxidative preconditioning' (Bilban *et al.*, 2008; Vieira *et al.*, 2008) but could also stimulate oxidative stress-induced tissue damage. Quite why such actions of CO should be distinct from damage due to hypoxia/ischaemia [which also involves increased ROS production (Elias-Miro *et al.*, 2013)] is presently unclear. However, CO can also, for example, stimulate NO production (Lim *et al.*, 2005; Kim *et al.*, 2006), and production of both ROS and NO by CO can also increase oxidative/nitrosative stress through formation of peroxynitrite ($ONOO^-$; Halliwell and Gutterridge, 2007).

In the heart, cardiotoxic effects of CO arise not only from ischaemic damage but also from its ability to cause endothelial damage and oxidative stress. In the short term, this can cause arrhythmias and, in the long term, following myocardial cell death, lead to cardiac fibrosis (Gandini *et al.*, 2001; Lippi *et al.*, 2012). Richard and colleagues (Andre *et al.*, 2010; Reboul *et al.*, 2012) and others (Gandini *et al.*, 2001) have provided much evidence that chronic exposure to CO levels leads to adverse cardiac remodelling. Importantly, levels of CO used experimentally for such chronic studies are comparable with those that can be experienced due to heavy traffic pollution or as a result of active or passive tobacco smoke inhalation (Reboul *et al.*, 2012). Cardiac remodelling by chronic CO exposure includes altered Ca^{2+} homeostasis, uncoupling of endothelial NOS (eNOS) and pro-arrhythmic changes in cardiac electrophysiology (Reboul *et al.*, 2012).

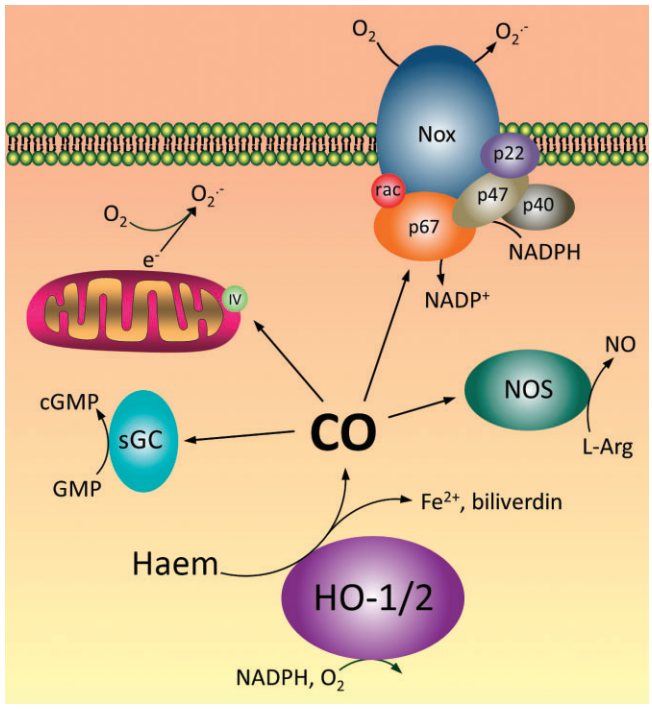

Figure 1

Established cellular targets of CO. Schematic showing CO, generated by degradation of haem by haem oxygenase-1 and -2 (HO-1/2), and the known cellular targets directly modulated by CO. These include the heteromultimeric NADPH oxidase, complex IV of the mitochondrial electron transport chain, sGC and NOS. Not shown is the MAPK pathway, since no specific target within this cascade has been identified as a target for CO.

Sub-lethal CO damage to the CNS can involve delayed neurological and neuropsychiatric symptoms (Min, 1986; Prockop and Chichkova, 2007; Piantadosi, 2008), and a significant fraction of patients are left with prolonged, if not irreversible, disabling neuronal damage, or encephalopathy (Gorman *et al.*, 2003; Mannaioni *et al.*, 2006). Necrotic damage of the iron-rich globus pallidus is commonly reported, possibly because of its relatively poor blood supply and hence greater vulnerability to ischaemia (Prockop and Chichkova, 2007), although damage to the cortex, hippocampus and temporal lobe is also frequently documented (Lo *et al.*, 2007). *Post-mortem* neuropathological studies reveal CO poisoning as the cause of infarctions and necrosis (Prockop and Chichkova, 2007), whereas experimental toxic CO exposure *in vivo* can trigger oxidative damage in rats (as evidenced by elevated malondialdehyde levels) and promote apoptosis, as suggested by elevated caspase 3 levels (Guan *et al.*, 2009). As in the myocardium, CO-triggered oxidative stress can lead to disturbances in Ca^{2+} homeostasis by triggering excessive influx, release from stores, or disrupting

buffering capabilities. This, in turn, can trigger deleterious downstream actions such as caspase activation and apoptosis, as reported in the complex processes underlying neurodegeneration associated with ageing or diseases such as Alzheimer's disease (Kruman and Mattson, 1999; Mattson, 2007; Bezprozvanny and Mattson, 2008; Green and LaFerla, 2008).

Beneficial effects of CO

Although it is poorly recognized in the public domain that CO is an influential, endogenous signalling molecule, documentation that living organisms can generate CO dates back over 100 years (detailed in Sjostrand, 1970). The source (haem) and degrading enzyme, which degrades haem to form endogenous CO (haem oxygenase; HO), was established almost 50 years ago (Tenhunen *et al.*, 1968; 1969). As shown in Figure 1, HO degrades haem, using oxygen and NADPH as co-factors, to produce biliverdin (rapidly converted into bilirubin via biliverdin reductase), free ferrous iron (Fe^{2+}) along with CO. Although both forms of HO perform this reaction, HO-1 differs from HO-2 in being inducible, rather than constitutively active. Regardless, the reaction is important for a number of reasons: it is a major means of recycling iron, and removal of pro-oxidant haem is also protective against oxidative stress. Furthermore, biliverdin and bilirubin are potent antioxidant agents in their own right (Stocker, 2004). However, our focus here is on the beneficial actions of CO.

In the heart (both the myocardium and the coronary circulation), as in other tissues, HO-1 induction occurs as an important part of myocardial responses to stress, including ischaemia/reperfusion (I/R) injury and infarction (Maulik *et al.*, 1996; Lakkisto *et al.*, 2002). This is clearly a protective action, since I/R injury is exacerbated in HO-$1^{+/-}$ mice (Yoshida *et al.*, 2001), and overexpression of HO-1 specifically in the myocardium protects against the same challenge (Yet *et al.*, 2001). Such protective effects of HO-1 are clearly at least partially mediated by CO since its administration [commonly via the use of CO-releasing molecules (CORMs), developed by Motterlini and co-workers (Motterlini *et al.*, 2002; Motterlini, 2007)] mimics the effects of HO-1 induction or overexpression, providing protection against I/R injury (Clark *et al.*, 2003; Guo *et al.*, 2004) and dilating coronary blood vessels (Musameh *et al.*, 2006).

Several studies point to CO as providing protection in the CNS. HO-1 can be induced in both neurones and glia (particularly astrocytes) in response to oxidative stress, ischaemic insult, excess glutamate and physical damage, and its up-regulation is also documented in neurodegenerative diseases such as Alzheimer's disease (Pappolla *et al.*, 1998; Dennery, 2000; Schipper *et al.*, 2009). HO-1 up-regulation appears protective, and this probably occurs at least in part because of the formation of CO: administration of exogenous CO has been shown to reduce the CNS damage associated with experimental focal ischaemia (Zeynalov and Dore, 2009). It has been proposed that specific up-regulation of HO-1 in astrocytes protects nearby neurones via CO production (Imuta *et al.*, 2007). Furthermore, CO has been shown to protect astrocytes from oxidative stress by altering their metabolic profile (Almeida *et al.*, 2012). The constitutively active HO-2 can also provide neuroprotection and studies suggest that this is also due specifically to the formation of CO by HO-2 (Dore *et al.*, 1999). At the cellular level, we have shown that oxidant-induced apoptosis can be markedly suppressed by CO, as detailed later (Dallas *et al.*, 2011; Al-Owais *et al.*, 2012).

It is clear from the above-described studies that the majority of the deleterious effects of CO arise from inhalation of exogenous CO, whereas most beneficial effects appear to be derived from endogenous CO. From a clinical perspective, the challenge for the future is to develop therapeutic approaches wherein exogenous CO can be beneficial, primarily by mimicking effects of endogenous CO, while avoiding the recognized deleterious effects of exogenous CO, associated with toxicity. Clearly, progress is being made in this regard (see Motterlini and Otterbein, 2010), yet our understanding of the diverse effects of CO – beneficial or otherwise – is incomplete.

Signalling pathways mediating cellular effects of CO

The biological activity of CO depends (seemingly exclusively) on its ability to interact with transition metals: there are no compelling data to suggest that it reacts chemically in any other manner within biological systems (Boczkowski *et al.*, 2006; Foresti and Motterlini, 2010; Motterlini and Otterbein, 2010). Since transition metals, including nickel, copper, cobalt and more commonly iron, are found within numerous diverse haem- and non-haem proteins, the potential for CO to modulate various signalling pathways is great; Figure 1 schematically summarizes some of the main pathways that have been shown to mediate many of the actions of CO. These directly involve known metal-binding (haem or haem-like) proteins or are presumed to be indirectly modulated by, as yet unidentified, metal-binding proteins.

CO can regulate intracellular ROS via a number of mechanisms; its ability to bind to complex IV (cytochrome *c* oxidase) of the mitochondrial electron transport chain can promote upstream electron leak, permitting formation of superoxide ions (Zuckerbraun *et al.*, 2007; Peers and Steele, 2012). CO can also uncouple mitochondrial respiration, suggesting that our understanding of its interaction with mitochondria is incomplete (Lo *et al.*, 2011). The NADPH oxidase (Nox) family of proteins, which are a widely distributed source of ROS required in numerous signalling pathways, can also be inhibited by CO, with significant consequences: for example, inhibition of Nox2 contributes to inhibition of airway smooth muscle proliferation (Taille *et al.*, 2005). Soluble guanylate cyclase (sGC) has long been known to be activated by CO (Kharitonov *et al.*, 1995), albeit at a much lower affinity than NO, leading to the production of cGMP. However, it should be noted that others have reported a failure of CO to act in this regard (Burstyn *et al.*, 1995). CO can also bind to NOS, thereby regulating NO formation. In some cases, this has been shown to be inhibitory (White and Marletta, 1992), but evidence also supports an activating role for CO in NO formation (Lim *et al.*, 2005). Although the underlying mechanism(s) and specific molecular targets involved are unknown, there is a significant body of evidence to indicate that CO can also interfere with MAPK signalling

(Kim *et al.*, 2006; Ryter *et al.*, 2006). Activation of p38 MAPK by CO may involve upstream MAP kinase kinase-3 (Otterbein *et al.*, 2000) or may be a less direct modulation, involving regulation of phosphatases or sGC activation (reviewed by Boczkowski *et al.*, 2006).

Ion channels as targets for the actions of CO

The pathways susceptible to modulation by CO summarized in Figure 1 are by no means exhaustive, and for simplicity do not highlight any interactions of pathways (such as modulation of both NO and ROS levels leading to the formation of peroxynitrite). They serve instead to illustrate some of the numerous possible mechanisms by which CO can regulate ion channels, and thereby exert many of its diverse beneficial and deleterious effects. These are discussed below, grouping ion channels according to their ion specificity for convenience and conforming to the *British Journal of Pharmacology's Concise Guide to PHARMACOLOGY* (Alexander *et al.*, 2013a). In many studies described, cells and channels have been exposed to CO by application of CORMs. These are valuable experimental tools and potential therapeutic agents pioneered and generously shared among researchers by Motterlini and colleagues (Motterlini *et al.*, 2002; Motterlini, 2007; Motterlini and Otterbein, 2010). However, some of their actions can occur independently of CO release (see, e.g. Wilkinson and Kemp, 2011b), and so judicial use of appropriate control compounds, as well as comparison of their effects with those of CO diluted directly into solution, should be performed wherever experimentally possible. For convenience, experimental exposure to such agents is referred to simply as CO exposure.

BK_{Ca} channels

Several research groups have studied the regulation of high conductance, Ca^{2+}-dependent K^+ channels [Slo1 (KCNMA1), variously termed $K_{Ca}1$, BK_{Ca} or maxiK channels] by CO (Hou *et al.*, 2009; Wilkinson and Kemp, 2011a). Physiologically, regulation of BK_{Ca} channels is significant as it has been proposed as a means by which CO can cause, for example, vasodilatation (Wang and Wu, 1997), or can control O_2 sensing by carotid body chemoreceptors (Williams *et al.*, 2004). There is unanimous agreement among different research groups that CO increases BK_{Ca} channel activity, but there is a distinct lack of consensus as to the molecular basis of how this increase in activity arises, despite a number of detailed investigations. Indeed, some findings are contradictory; for example, CO has been proposed to mimic the ability of Ca^{2+} to activate this channel (Hou *et al.*, 2008), yet others have shown that CO stimulates channel activity even when Ca^{2+} is saturating (Williams *et al.*, 2008), and fails to do so in the absence of Ca^{2+} (Telezhkin *et al.*, 2011). Similarly, mutagenesis studies (e.g. Williams *et al.*, 2008) have discounted previously proposed extracellular histidine residue(s) as mediating effects of CO (Wang and Wu, 1997). Most strikingly, Jaggar *et al.* (2005) provided compelling evidence to indicate that CO regulates BK_{Ca} channels by binding specifically to reduced haem, thereby disrupting its interaction with the channel at a conserved haem-binding domain. These workers mutated a histidine and cysteine residue within this domain and found that CO no longer activated the channel. However, others have shown that mutation of the same histidine residue necessary for haem binding did not alter CO sensitivity (Hou *et al.*, 2008; Williams *et al.*, 2008), and that CO sensitivity was also independent of redox status (Hou *et al.*, 2008).

Given this body of seemingly contradictory data, combined with the likely possibility that CO somehow interacts directly with the BK_{Ca} channel, Kemp and co-workers considered alternative (non-haem) metal-binding structures as potential sites within BK_{Ca} for CO interaction. They demonstrated that cyanide (known to interact with metal 'cluster' sites in other proteins) could prevent channel activation by CO, and that CO sensitivity was dramatically reduced after substitution of a cysteine residue in the C-terminal domain (Telezhkin *et al.*, 2011). Their findings are consistent with their idea that a metal-containing, non-haem structure, linked to the channel via cysteine thiol groups, may act as a CO interaction site. Such cyanide-sensitive structures have previously been identified in other proteins and are worthy of further exploration as potential sites of direct modulation by CO particularly in BK_{Ca} (where alternative models appear contradictory), but also in other channel proteins where direct interaction with CO is considered likely.

$K_v2.1$ channels

The voltage-gated delayed rectifier K^+ channel $K_v2.1$ (KCNB1) is unusual among K^+ channels in being regulated in an exquisitely sensitive manner through phosphorylation by various kinases acting at numerous identified sites (Park *et al.*, 2006; Mohapatra *et al.*, 2009). Phosphorylation status strongly influences the channel's voltage-dependence and kinetics and, in so doing, dramatically alters excitability of central neurones; $K_v2.1$ is particularly highly expressed in somatodendritic regions of hippocampal and cortical neurones where it strongly influences excitability during periods of high frequency firing (Murakoshi and Trimmer, 1999; Du *et al.*, 2000). $K_v2.1$ has also been strongly implicated as a route through which neurones can become depleted of cellular K^+ as an early step in the process of oxidative stress-induced apoptosis (Yu, 2003). Specific involvement of $K_v2.1$ in apoptosis has been demonstrated in cortical neurons, and introduction of the channel into CHO cells increases apoptosis in response to oxidative stress (Pal *et al.*, 2003; 2006). In response to oxidants, $K_v2.1$ channels are inserted into the plasma membrane in a process that is tightly regulated by phosphorylation of the channel at Ser^{800} under the control of p38 MAPK (Redman *et al.*, 2007). Phosphorylation at the N-terminal Y124, controlled by Src kinase activity, is also required for channel insertion into the membrane (Redman *et al.*, 2009). Coordination of this mechanism is determined by functionally independent rises of $[Ca^{2+}]_i$ and $[Zn^{2+}]_i$ triggered by the initial oxidative stress (McCord and Aizenman, 2013).

As discussed earlier, HO-1 is up-regulated in the CNS following oxidative stresses associated with, for example, stroke or neurodegenerative diseases, and both HO-1 and HO-2 provide neuronal protection under such circumstances (Ferris *et al.*, 1999; Dore *et al.*, 2000; Ahmad *et al.*, 2006). Given that CO inhalation is neuroprotective against experimental stroke (Zeynalov and Dore, 2009), and that CO derived from astrocytes in response to hypoxia can protect neighbouring neurons from apoptosis (Imuta *et al.*, 2007), we explored the possibility that CO regulation of $K_v2.1$ may be involved in its neuroprotective actions. CO reversibly inhibited recombinant $K_v2.1$ expressed in HEK293 cells in a manner that did not alter its voltage-dependence, distinguishing its inhibitory effects from those of dephosphorylation (Dallas *et al.*, 2011). The mechanism of inhibition was not fully elucidated, but depended in part on increased mitochondrial ROS formation. Although NO formation was discounted as a possible contributory factor, CO was only effective when the channel was tonically phosphorylated by PKG (Dallas *et al.*, 2011). Although the mechanism of CO inhibition of $K_v2.1$ remains to be elucidated fully, the consequences of channel inhibition were clear: expression of $K_v2.1$ in HEK293 cells increased their vulnerability to oxidative stress-induced apoptosis, and this was largely inhibited by CO (Dallas *et al.*, 2011). More importantly, CO also provided protection against oxidative stress-induced apoptosis in primary cultures of hippocampal neurones, fully inhibited the oxidant-induced increase in whole-cell K^+ current and showed at least partial selectivity in its ability to inhibit $K_v2.1$ in these cells. These findings provide a candidate mechanism by which CO (and perhaps also increased HO-1 expression) might provide neuroprotection against damaging insults, and further supports the idea that $K_v2.1$ is of central importance in this process.

K_{2P} channels

Two pore-domain K^+ channels (K_{2P} channels) are an important and widely distributed family of K^+ channels. They comprise subunits of four transmembrane domains and two pore-forming domains that form constitutively active channels as homo- or heteromeric dimers. Their constitutive activity exerts a major influence on cell excitability, particularly but not exclusively in central neurones, and their sensitivity to various physiological and pharmacological modulators largely accounts for neuronal responses to, for example, temperature, pH, fatty acids and volatile anaesthetics (Plant *et al.*, 2005; Mathie *et al.*, 2010). Perhaps the best studied to date, at least within the context of the CNS, is the mechano-sensitive TREK-1 ($K_{2P}2.1$; KCNK2), the activity of which is acutely influenced by membrane stretch, lipids, GPCR agonists as well as the above-named factors. Such polymodal regulation, combined with its widespread distribution, results in this channel exerting important influences on a wide range of neuronal functions (Honore, 2007).

To date, three subtypes of K_{2P} channel have been explored in terms of sensitivity to CO, all using heterologous expression systems. Currents generated in HEK293 cells expressing human acid-sensing K_{2P} channels TASK-1 and TASK-3 were unaffected by CO (Dallas *et al.*, 2008). By contrast, recombinant human TREK-1 ($K_{2P}2.1$) expressed in HEK293 cells was reversibly increased in amplitude on exposure to lower levels of CO. However, current augmentation diminished with increasing CO concentration, and CO was inhibitory at higher concentrations (Dallas *et al.*, 2008). Interestingly, both effects of CO (augmentation and inhibition) were mimicked by exposure of cells to NO, yet the effects of CO were not mediated by NO formation, since they were apparent in the presence of an NO scavenger and during inhibition of NO formation (Dallas *et al.*, 2008). However, CO was ineffective during PKG inhibition, consistent with the involvement of sGC activation. Compelling evidence indicates that TREK-1 in the CNS plays a major role in nociception, neuroprotection against glutamate excitotoxicity, general anaesthesia and mood regulation (Honore, 2007). Such roles may also be influenced by CO exposure/HO expression, yet at present remain largely unexplored.

Na^+ channels

Despite the proposed beneficial effects of CO as a therapeutic approach to lung disease and acute lung injury (Ryter and Choi, 2006), little was known about the effects of CO on fundamental aspects of lung physiology, such as alveolar fluid clearance, until the study of Althaus *et al.* (2009). These workers investigated the effects of CO on alveolar fluid reabsorption in the isolated rabbit lung, and observed a reduction in fluid clearance due to inhibition of amiloride-sensitive Na^+ transport. Consistent with this observation was the finding that CO inhibited amiloride-sensitive, transepithelial currents in a human lung epithelial cell line and in rat alveolar cells, and this effect was attributed to inhibition of the apical Na^+ channel, ENaC. sGC, cGMP and ROS were discounted as mediators of this effect of CO, and instead, it was suggested that CO may interact with histidine residues on one or more ENaC subunits or associated proteins, since chemical modification of histidine residues (via application of diethyl pyrocarbonate, as employed in studies of BK_{Ca} channels, see earlier section) disrupted CO modulation of ENaC. Wang *et al.* (2009) used excised membrane patches to investigate the effects of CO on ENaC in cultured collecting duct cells from murine kidney cortex at the single channel level. In contrast to the study of Althaus *et al.* (2009), they found that CO increased ENaC activity and proposed that ENaC regulation may be controlled by CO derived from localized haem degradation, as previously described for BK_{Ca} channels (Williams *et al.*, 2004), although in this case no evidence for co-localization of a haem oxygenase with ENaC was provided. More confounding, however, is the fact that opposing effects of CO on ENaC have been reported. This is not unprecedented in the field (see section on Ca^{2+} channels), but requires resolution before a full understanding of the ENaC-mediated effects of CO on epithelial transport can be achieved, and hence whether such effects may be diverse according to tissue type, or due to artefactual differences in channel properties arising from unrecognized differences in experimental conditions.

Voltage-gated Na^+ channels are a major factor in determining the excitability of nerves, cardiac and skeletal muscle and other tissues, providing the rapid upstroke of the action

potential (Catterall, 2012). In the heart, $Na_v1.5$ is the dominant channel type, its major pore-forming α subunit encoded by SCNA5, 1 of 10 genes giving rise to this class of ion channel. Mutations in this channel account for many types of arrhythmias, such as Brugada syndrome and long QT arrhythmias (Amin *et al.*, 2010; Andavan and Lemmens-Gruber, 2011). Interestingly, a number of case reports published over several decades have noted arrhythmia-like events in patients hospitalized due to CO exposure, suggesting that CO can disrupt cardiac excitability (Peers and Steele, 2012). To explore this, we recently studied the effects of CO in isolated ventricular myocytes and noted that CO caused a dramatic prolongation of the cardiac action potential and associated Ca^{2+} transient; in many instances this was associated with early after depolarization-like arrhythmias, strikingly similar to those associated with long QT-3 (LQT-3) syndrome (Dallas *et al.*, 2012). Voltage-clamp recordings revealed that CO inhibited the peak Na^+ current and, more importantly, increased the amplitude of the late Na^+ current. This latter effect is reminiscent of the effects of a number of SCNA5 mutations, which give rise to LQT-3-like arrhythmias (Amin *et al.*, 2010).

One unusual group of patients with LQT-3-like arrhythmias actually express non-mutant forms of $Na_v1.5$, but instead have mutations in the associated protein syntrophin (Ueda *et al.*, 2008), which is part of a macromolecular complex incorporating $Na_v1.5$, a plasmalemmal Ca^{2+} ATPase and also nNOS. Patients with syntrophin mutations tonically generate increased levels of NO within this complex, which nitrosylates $Na_v1.5$ thereby increasing the amplitude of the late Na^+ current and hence causing arrhythmias similar to those observed in patients with LQT-3 syndrome arising from SCNA5 mutations (Ueda *et al.*, 2008). This complex is probably involved in the actions of CO on $Na_v1.5$, as illustrated in Figure 2, since the actions of CO to induce arrhythmias, and increase the late Na^+ current, were mimicked by NO donors and prevented by inhibiting NO formation (Dallas *et al.*, 2012). Furthermore, CO exposure led to nitrosylation of the $Na_v1.5$ protein. The pro-arrhythmic effects of CO were observed *in vivo*, when rats were exposed to 500 ppm CO and ECG measurements monitored by telemetry. Furthermore, when injected with isoprenaline during CO exposure, most animals experienced ventricular tachycardia, and some developed fatal ventricular arrhythmias (Dallas *et al.*, 2012). This finding is somewhat ominous, since this level of CO exposure is only slightly higher than levels detected in urban pollution (Reboul *et al.*, 2012). Of clinical significance was the observation that ranolazine, an anti-anginal agent known to inhibit the late Na^+ current (Saint, 2008), largely reversed the pro-arrhythmic effects of CO *in vitro* and *in vivo* (Dallas *et al.*, 2012), suggesting that it may be useful as an immediate therapy for cardiac arrhythmias associated with CO poisoning. This study is, to our knowledge, the first to identify an ion channel as a target for modulation by CO as part of its toxic rather than physiological actions.

Voltage-gated Ca^{2+} channels (VGCCs)

A small number of groups have independently explored the effects of CO on voltage-gated L-type Ca^{2+} channels, with

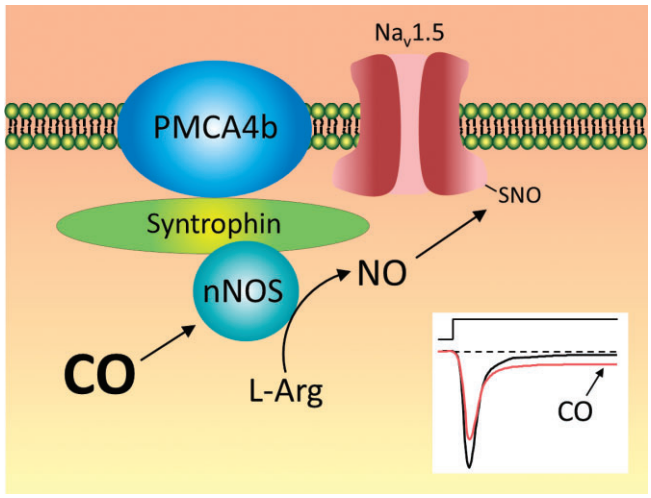

Figure 2

CO induces the late cardiac Na^+ current. The cardiac Na^+ channel $Na_v1.5$ forms part of a macromolecular complex that also incorporates a plasma membrane Ca^{2+}-ATPase (PMCA4b), syntrophin and nNOS (Ueda *et al.*, 2008). CO increases nNOS activity, generating a localized increase in NO levels, which modulates $Na_v1.5$ through nitrosylation. This modification causes an increase in the amplitude of the late Na^+ current (Dallas *et al.*, 2012). The inset shows a schematic of Na^+ currents evoked in a voltage-clamped cardiac myocyte by step depolarizations. Note that in the presence of CO (red trace), the peak amplitude is reduced, but the late current amplitude is increased.

surprisingly varied effects (Table 1). Two groups have reported inhibition of currents in cardiac or cardiac-derived tissue (Uemura *et al.*, 2005; Scragg *et al.*, 2008), whereas others have reported a modest but significant augmentation of currents recorded in human jejunal smooth muscle cells (Lim *et al.*, 2005). Inhibition of cardiac myocyte L-type Ca^{2+} currents is mediated by CO-induced increases in mitochondrial ROS formation (Scragg *et al.*, 2008), whereas augmentation of jejunal smooth muscle currents is, by contrast, mediated by increased formation of NO and activation of cGMP [but not PKG; instead a role for PKA is implicated (Lim *et al.*, 2005)]. Such diverse responses and underlying mechanisms are conceivable, given the different tissues studied and hence the probable different coupling and/or localization of signalling pathway components that might mediate any effects of CO on these native channels. However, a surprising observation was that these differential effects – and the associated underlying signalling pathways – were also seen in very similar recombinant expression systems: thus, transient expression of $Ca_v1.2$ cloned from human jejunum (together with a β_2 subunit) generated currents, which were modestly augmented by CO in a NO- and cGMP-dependent manner (Lim *et al.*, 2005). By contrast, human cardiac $Ca_v1.2$ channels, stably expressed in HEK 293 cells in the absence of auxiliary subunits, were inhibited by CO in a manner that was dependent on the increased generation of mitochondrial ROS. Mutagenesis studies identified three key cysteine residues in the C-terminal domain as necessary for such inhibition (Scragg *et al.*, 2008). Whether or not such striking differences

Table 1

Published effects of CO on L-type Ca^{2+} channels

Study	Preparation	Effect of CO	Mechanism
Uemura *et al.* (2005)	Embryonic cardiac myocytes cell line, H9c2	Inhibited current (60%)	Not determined
Lim *et al.* (2005)	Human jejunal smooth muscle cell, perforated patch recording	Increased current (14%)	Increased NO and cGMP, but not PKG (possibly PKA)
Lim *et al.* (2005)	Transient expression of human jejunal $Ca_v1.2$ together with β_2 subunit in HEK293 cells	Increased current (20%)	Increased NO and cGMP, but not PKG (possibly PKA)
Scragg *et al.* (2008)	Adult rat ventricular myocytes	Inhibited current (60%)	Increased production of mitochondrial ROS
Scragg *et al.* (2008)	Human cardiac $Ca_v1.2$ expressed stably in HEK293 cells	Inhibited current (60%)	Increased production of mitochondrial ROS acting at C-terminal cysteine residues

Summary table indicating the reported effects of CO on native and recombinant L-type Ca^{2+} channels, and the proposed mechanisms (where investigated) underlying such regulation.

in the reported responses to CO are attributable to auxiliary subunits, expression protocols or any undetermined structural differences in the α subunits employed in these studies remain to be determined and require further investigation.

T-type Ca^{2+} channels are unique among VGCCs, being distinguished by their kinetic and pharmacological properties and because they are activated at voltages below the threshold for other VGCCs (Carbone and Lux, 1984; Perez-Reyes, 2003; Iftinca and Zamponi, 2009). Three genes (*CACNA1G*, *CACNA1H* and *CACNA1I*) encode T-type Ca^{2+} channels, giving rise to voltage-sensing, pore-forming subunits, termed $Ca_v3.1$–3.3 (Catterall *et al.*, 2005). Heterologous expression of these genes produces currents similar to native currents, implying channel function is determined by the α subunits alone, without a strong requirement for auxiliary subunits. A recent study has demonstrated that CO regulates all three T-type Ca^{2+} channels when expressed in HEK293 cells, with similar potency (Boycott *et al.*, 2013). Interestingly, however, the mechanism underlying CO inhibition varies between channel isoforms: detailed studies discounted known pathways of modulation (illustrated in Figure 1) for $Ca_v3.2$ and, instead, revealed a novel mechanism by which this channel is regulated. Probing the redox sensitivity of $Ca_v3.2$, Boycott *et al.* (2013) found that $Ca_v3.2$ was regulated tonically by thioredoxin (Trx-1) acting at an extracellular site. Although not unprecedented (Xu *et al.*, 2008), this unusual means of redox modulation is dependent on transmembrane transport of reduced Trx-1 via an unknown pathway to act extracellularly in order to tonically increase channel activity. CO was found to interrupt this pathway, although the point of interruption was not identified: candidate sites at which regulation could be interrupted are shown in Figure 3. Intriguingly, the involvement of Trx-1 in CO inhibition of $Ca_v3.1$ and $Ca_v3.3$ was discounted, and the mechanisms underlying their regulation by CO remain to be determined. T-type Ca^{2+} channels are involved in biological processes as diverse as nociception (Todorovic and Jevtovic-Todorovic, 2011) and cellular proliferation (Santoni *et al.*, 2012). Thus, via inhibition of these channels, CO is likely to be influential in these processes. Future studies will determine the extent of this influence.

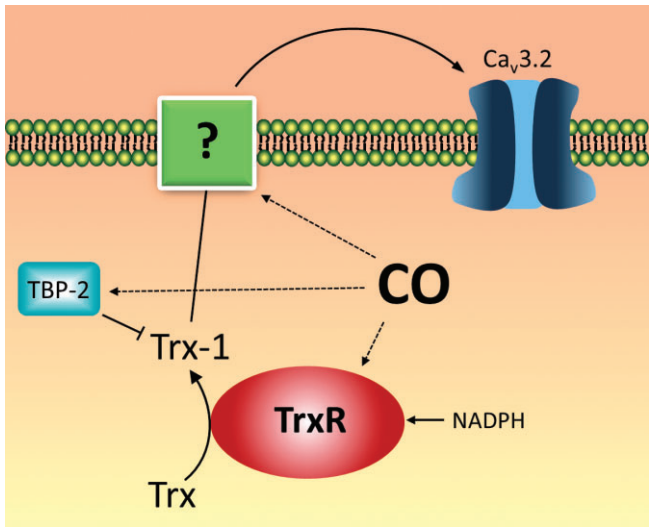

Figure 3

Putative mechanisms for the inhibition of $Ca_v3.2$ T-type Ca^{2+} channel by CO. Cartoon depicting the regulation of the $Ca_v3.2$ T-type Ca^{2+} channel by the thioredoxin system. Thioredoxin reductase (TrxR) 'recycles' thioredoxin (Trx) into its reduced form (Trx-1) using NADPH. Trx-1 is negatively regulated by Trx binding protein-2 (TBP-2), also known as vitamin D up-regulated protein-1 (VDUP-1) and Trx interacting protein (TXNIP). It can also be transported out of cells via an unknown mechanism (depicted by green box) to act extracellularly in the regulation of $Ca_v3.2$ (Boycott *et al.*, 2013). CO inhibits $Ca_v3.2$ via disruption of thioredoxin regulation, but the site at which this occurs is currently unknown. Candidate targets are indicated.

P2X2 receptors

Ligand-gated ion channels represent a large family of ion channels (for nomenclature see Alexander *et al.*, 2013b) and remain largely unexplored in terms of their sensitivity to CO. The one exception is the P2X receptor group, which forms cation-permeable channels activated by extracellular ATP,

particularly the P2X2 subtype. Wilkinson *et al.* (2009) demonstrated that native and recombinant homomeric P2X2 receptors were reversibly augmented by CO in the presence of low ATP concentrations. The effects were strikingly rapid and potent, and also highly selective: a lack of effect, or modest inhibition, was reported for P2X2/3 heteromers, P2X3 and P2X4 receptors. The mechanism by which CO augmented P2X2 receptors was not elucidated, but the involvement of sGC or cGMP was discounted (Wilkinson *et al.*, 2009). Perhaps more importantly, CO regulation of these channels suggests that CO may be influential as a signalling molecule in a number of previously unrealized, diverse physiological processes, such as nociception (North, 2002).

Concluding remarks

Evidence is clearly accumulating that ion channels represent an important family of target proteins for CO. It is apparent that their modulation contributes to many of the physiological and therapeutic actions of CO, as well as to some of its toxic effects. Equally apparent, however, is the limited knowledge we have of this field currently: many ion channel families (particularly ligand-gated ion channels) have yet to be explored in terms of their sensitivity to CO, and the coming years will probably reveal numerous more target channels. Given the widespread distribution of haem oxygenases, such findings will doubtless be of physiological significance. Furthermore, ion channel regulation by CO can also be subject to signalling cross-talk between CO and other gasotransmitters (namely NO and H_2S), as already evidenced, for example, in the process of O_2 sensing in the carotid body chemoreceptor (Prabhakar and Peers, 2014). Understanding the various mechanisms by which channels are regulated by CO is equally important if we are to benefit from its potential therapeutic actions, and distinguish them from mechanisms underlying its toxicity. Unfortunately, the field has already thrown up areas of contention and lack of consensus regarding some of the means by which CO can regulate channel activity. Such discrepancies must be rectified before we can fully exploit the potential benefits of this gasotransmitter, or understand and so counteract the detrimental effects of this potent toxin.

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Conflict of interest

None.

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