The RCK1 high-affinity Ca^{2+} sensor confers carbon monoxide sensitivity to Slo1 BK channels

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Carbon monoxide (CO) is a lethal gas, but it is also increasingly recognized as a physiological signaling molecule capable of regulating a variety of proteins. Among them, large-conductance Ca^{2+} - and voltage-gated K^{+} (Slo1 BK) channels, important in vasodilation and neuronal firing, have been suggested to be directly stimulated by CO. However, the molecular mechanism of the stimulatory action of CO on the Slo1 BK channel has not been clearly elucidated. We report here that CO reliably and repeatedly activates Slo1 BK channels in excised membrane patches in the absence of Ca^{2+} in a voltage-sensor-independent manner. The stimulatory action of CO on the Slo1 BK channel requires an aspartic acid and two histidine residues located in the cytoplasmic RCK1 domain, and the effect persists under the conditions known to inhibit the conventional interaction between CO and heme in other proteins. We propose that CO acts as a partial agonist for the high-affinity divalent cation sensor in the RCK1 domain of the Slo1 BK channel.

maxi K | potassium channel | heme | oxygen sensing | $\text{K}_{\text{Ca}1.1}$

Carbon monoxide (CO) is a deadly poisonous gas. However, CO is physiologically produced during the course of heme catabolism by heme oxygenases (HMOXs) in an oxygen-dependent manner, and it is increasingly recognized as a biological signaling molecule important in numerous physiological and pathophysiological processes, including synaptic plasticity, regulation of vascular tone, and tumor proliferation (1). To regulate the wide array of cellular functions, CO typically exerts its action by binding to the reduced heme iron center (Fe^{2+}) in hemoproteins (2, 3), thereby altering the way the heme prosthetic group is coordinated (4). The heme-dependent action of CO is exemplified by its stimulatory effect on soluble guanylate cyclase, in which binding of CO to the reduced heme iron center increases the catalytic activity, leading to an enhanced production of cGMP (4, 5).

Large-conductance Ca^{2+} - and voltage-activated K^{+} (Slo1 BK or $\text{K}_{\text{Ca}1.1}$) channels, important in many physiological phenomena, including oxygen sensing, vasodilation, and neuronal firing (6, 7), are also stimulated by CO (8–12). The modulation of Slo1 BK channels by CO is physiologically and pathophysologically important. For example, the stimulatory effect of CO on Slo1 BK channels underlies its well documented vasodilatory effect (9, 11, 13–15), and the use of CO has been suggested as a potential therapeutic strategy against pulmonary hypertension (11). Production of CO by HMOXs requires oxygen (1), and this oxygen dependence raises the possibility that changes in cellular oxygen tension regulate the Slo1 BK channel activity indirectly by regulating the availability of CO (16). HMOX-2, one member of the HMOX family, may colocalize with Slo1 to allow efficient modulation of the channel by CO according to the local oxygen level (16).

Gating of the Slo1 BK channel involves allosteric interactions among the main pore gate, voltage sensor domains, and cytoplasmic RCK1 and RCK2 domains with multiple divalent cation sensors (17, 18). Although increasing evidence suggests that the Slo1 BK channel is directly stimulated by CO without any

requirement for a cGMP-dependent signaling pathway (8, 10), how the allosteric gating mechanism of the Slo1 BK channel is directly altered by CO has not been clearly elucidated. The finding that heme is capable of acutely binding to the Slo1 BK channel and often inhibits the channel activity (19, 20) prompted Jaggar *et al.* (21) to examine whether the reversal of the inhibitory effect of heme by CO underlies the stimulatory action of CO on the Slo1 BK channel. Consistent with the widely held view that CO interacts only with reduced heme in hemoproteins (2, 3), it was suggested that CO reverses the inhibitory effect of heme on the channel only under a reducing condition (21).

In this study, we investigated the mechanism of the direct stimulatory effect of CO on the Slo1 BK channel with an emphasis on the biophysical mechanism and the redox dependence. Our results show that CO acts as a partial agonist for the high-affinity divalent cation sensor in the RCK1 domain in a redox-independent manner and that two histidines and an aspartic acid in the high-affinity divalent cation sensor domain are required for the CO action.

Results

To elucidate the mechanism of the CO action, we applied CO gas or the CO donor reagent $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ (12, 16, 22) to heterologously expressed channels composed of the pore-forming subunit Slo1 alone, as well as those composed of Slo1 and $\beta 1$, the predominant auxiliary subunit in smooth muscle cells (23). In both channel types, CO gas and the CO donor consistently and repeatedly increased open probability in the virtual absence of Ca^{2+} in the cell-free excised patch configuration in a fully reversible manner without altering the unitary current amplitude [Fig. 1A and supporting information (SI) Fig. 5]. The current-enhancing effect of CO, up to 600%, was most prominently observed at physiologically relevant voltages and progressively became smaller with greater depolarization (Fig. 1B). In contrast with the robust stimulatory effect observed on Slo1 BK channels, CO was ineffective on SK2 small-conductance Ca^{2+} -activated K^{+} channels, which possess a Ca^{2+} -dependent activation mechanism distinct from that of the Slo1 BK channel (SI Fig. 6) (24). Nitric oxide (NO), another gaseous messenger, did not stimulate the Slo1 BK channel (SI Fig. 7).

We previously showed that heme, whether reduced or oxidized (hemin), profoundly alters the allosteric gating of the Slo1 BK channel (19, 20); heme decreases open probability at depolarized voltages but increases the probability at negative voltages. The modulatory effect of heme diminishes when the putative heme-binding sequence, $^{612}\text{CKACH}^{616}$, in the linker segment between the cytoplasmic RCK1 and RCK2 domains, is

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Fig. 2. The stimulatory effect of CO persists at negative voltages but diminishes at a saturating $[Ca^{2+}]_i$. (A) (Left) Representative macroscopic currents at 60 mV from the WT Slo1 channels, pretreated with DEPC before and after CO application in the absence of Ca^{2+} . DEPC (2 mM) was applied to the intracellular side typically for 5 to 10 min and washed out. (Right) Fractional increases in the peak outward current size after CO application at 100 mV in the control and DEPC-treated groups are also shown ($n = 5-8$). (B) Representative openings at -50 mV, -100 mV, and -150 mV before (Left) and after (Right) CO application in the absence of Ca^{2+} . Downward reflections represent opening transitions. Statistical information is presented in Fig. 1B Right (squares). (C) Representative macroscopic currents in the presence of Ca^{2+} . The currents were recorded at 80 mV (200 nM $[Ca^{2+}]_i$), -20 mV ($5 \mu M$ $[Ca^{2+}]_i$), and -90 mV ($120 \mu M$ $[Ca^{2+}]_i$) so that the normalized conductance under each condition is ≈ 0.125 . No leak or capacitive current subtraction was performed for the results at 5 and $120 \mu M$ $[Ca^{2+}]_i$.

Fig. 3. Select mutations in the RCK1 high-affinity Ca^{2+} sensor specifically abolish the CO sensitivity. (A) A schematic structural organization of a Slo1 polypeptide. Circles, histidines; triangle, D367. (B) Representative currents at 100 mV from H365 and H394 mutants (*Upper*) before (thin sweeps) and after (thick sweeps) CO application and the fractional increase in the peak current size by CO at different voltages (*Lower*) in the absence of Ca^{2+} . Circles represent data from macroscopic peak outward currents, and squares represent data from single-channel currents. The dashed curve in each graph represents the average data from the WT channel ($n = 4-8$). (C) (*Left*) Representative currents before and after CO application from the D367A channels at 80 mV. The mean fractional increase at this voltage was $1.5 \pm 10\%$. (*Right*) The lack of the CO effect at different voltages is also shown ($n = 4-6$). (D) Representative WT currents before and after CO application with 10 mM intracellular Mg^{2+} (*Left*) and currents at 100 mV from the E374A (*Center*) and E399A channels in the absence of Mg^{2+} (*Right*). The mean increases in the current size by CO in E374A and E399A at 100 mV were $147 \pm 40\%$ and $161 \pm 19\%$, respectively. All recordings were performed in the virtual absence of intracellular Ca^{2+} .

H365, required for the CO action, is located near D367, a probable high-affinity Ca^{2+} sensor in the RCK1 domain (27). Thus, we hypothesized that the functional interaction between CO and H365/H394 may influence the Ca^{2+} -sensing process mediated by D367. This idea, in turn, suggests the CO sensitivity of the channel may diminish when the Ca^{2+} sensing by D367 is impaired. The mutation D367A, which is known to interfere with the high-affinity Ca^{2+} -dependent activation of the Slo1 channel (27), rendered the channel insensitive to CO, thus suggesting that D367 is another essential component in the CO sensitivity (Fig. 3C).

In addition to the high-affinity Ca^{2+} sensor, the RCK1 domain also possesses a low-affinity divalent cation sensor, which includes E374 and E399; this sensor mediates Mg^{2+} -dependent activation of the Slo1 channel (27, 30). Unlike the interaction between CO and Ca^{2+} shown above, high concentrations of Mg^{2+} did not alter the efficacy of CO to stimulate the Slo1 channel activity, and the mutations E374A and E399A, which

disrupt the low-affinity sensor (27, 30), had no effect on the channel's sensitivity to CO (Fig. 3D).

The CO sensitivity of the channel is clearly disrupted by mutation of H365, H394, or D367 in the RCK1 Ca^{2+} sensor subdomain (Fig. 3B and C); however, these mutations failed to alter the sensitivity of the channel to hemin (Fig. 4A). Hemin, applied to the cytoplasmic side, markedly decreased the currents through the WT and the CO-insensitive mutant channels, indicating that the channel's sensitivities to CO and hemin are mediated by distinct mechanisms. In other CO-sensitive proteins, CO interacts with the reduced heme iron (Fe^{2+}) (2, 3), and it is well established that the interaction of CO with heme is inhibited by oxidation of the iron center (3). However, treatment of the Slo1 channel with the oxidant H_2O_2 (0.07%), which easily oxidizes the heme iron in myoglobin, as verified spectrophotometrically (SI Fig. 10), did not alter the efficacy of CO on the Slo1 channel (Fig. 4B). The CO action is therefore independent

Fig. 4. CO and hemin affect the Slo1 channel activity through distinct mechanisms. (A) Inhibitory effects of hemin on the currents from the WT channel and the CO-insensitive Slo1 H365R, H394R, and D367A channels at 160, 100, 160, and 130 mV, respectively. Hemin (100 nM) was applied to the cytoplasmic side. $n = 3$ in each channel type. Similar results were obtained from the double mutants H365R:H394R and H365A:H394A. (B) Representative WT currents at 100 mV before and after application of CO after pretreatment with H_2O_2 (0.07%) for 10 min and wash. The increase in the peak outward current size was $106 \pm 11\%$ ($n = 3$). (C) Representative WT currents at 100 mV before and after application of CO in a patch pretreated with $\text{pH}_i = 3$ for 10 min followed by a wash with a normal internal solution ($\text{pH}_i = 7.2$). The mean peak outward current increase was $122 \pm 22\%$ ($n = 4$), respectively. All recordings were performed in the virtual absence of intracellular Ca^{2+} .

of the redox environment of the channel, and it is unlikely that easily oxidizable cofactors, such as Fe^{2+} or heme, are required. Furthermore, in many hemoproteins, heme cofactors can be removed by extended treatment with low intracellular pH (pH_i) (31). However, pretreatment with $\text{pH}_i = 3$ for 10 min, followed by wash, failed to eliminate the stimulatory effect of CO on the Slo1 channel (Fig. 4C).

Discussion

The gating mechanism of the Slo1 BK channel, which involves multiple allosteric interactions among the main gate, voltage sensors, and ligand sensors (18), is modulated by a variety of signaling molecules. Increasing evidence shows that CO stimulates the Slo1 BK channel without any requirement for cytoplasmic signaling cascades (16, 26). However, the biophysical and molecular mechanisms underlying the stimulatory action of CO remained unresolved. A previous study analyzing openings of native vascular BK channels suggested that CO increases the channel activity in part by shifting the overall Ca^{2+} dependence of activation to lower concentrations (10). The study also indicated that the stimulatory effect of CO decreases at lower $[\text{Ca}^{2+}]_i$. We have shown here that CO enhances Slo1 BK ionic currents in the virtual absence of intracellular Ca^{2+} without altering the unitary current size. Furthermore, because the increase in open probability by CO is maintained at very negative voltages, the CO action does not require activation of the voltage sensors. We therefore conclude that CO increases open probability by altering the intrinsic equilibrium of the main gate of the channel between the closed state and the open state or the equilibrium constant L_0 in the formulation of Horrigan and Aldrich (20).

Our results show that the stimulatory effect of CO on the channel's main gate is mediated by the RCK1 high-affinity Ca^{2+} sensor. The effect of CO is absent at saturating $[\text{Ca}^{2+}]_i$, and mutation of D367 abolishes the channel's sensitivity to CO, suggesting that CO functionally mimics the activating effect of Ca^{2+} on the channel. In this way, the effect of CO is independent of the voltage sensor activation and diminishes when the Ca^{2+} -mediated activation dominates at high $[\text{Ca}^{2+}]_i$.

Besides D367, two nearby histidine residues, H365 and H394, in the cytoplasmic RCK1 high-affinity Ca^{2+} sensor are also essential for the CO sensitivity of the Slo1 channel. This finding is consistent with an earlier suggestion that histidine may be important (26), although the original study implicated extracellularly accessible histidine residues. The reason for the histidine requirement is not entirely clear. Considering that histidine is a common ligand for heme binding in hemoproteins, one possibility is that H365 and/or H394 coordinate heme in the RCK1 domain and that CO binds to the heme iron center to alter the channel gating. We consider this scenario unlikely for the following reasons. First, the available high-resolution structures of the RCK domain of the bacterial channel MthK (32, 33), whose primary amino acid sequence is similar to that of the Slo1 RCK1 domain (34), do not show any bound heme. Second, the conventional interaction between CO and heme requires that the iron center is reduced (2, 3), but the stimulatory effect of CO persists in the excised patch configuration even after harsh treatment with the oxidant H_2O_2 . It is worth noting that in our ambient recording condition, the heme iron center in myoglobin is found fully oxidized (SI Fig. 10), confirming that the heme iron is readily oxidized. If the Slo1 channel contained any heme or iron, it would be oxidized under our experimental conditions and unable to interact with CO. Recently, some evidence that microbial proteins without cofactors may be able to interact with gases has surfaced (35). In these microbial gas-interacting proteins, histidine and aspartic acid residues are critical (35) while the effect of CO on the Slo1 channel requires H365, H394, and D367. Taken together, these findings lead us to speculate that

CO, with its unequal electron densities around the two atoms, may directly interact with the histidine and aspartic acid residues in the RCK1 high-affinity Ca^{2+} sensor, in part, using an electrostatic mechanism. Other physical forces must aid the interaction between CO and the RCK1 sensor because the dipole moment of CO is weak. The structural specificity of the RCK1 Ca^{2+} sensor for CO is, in part, evidenced by the inability of NO to exert a similar stimulatory effect.

The mechanism of CO sensing using the RCK1 high-affinity Ca^{2+} sensor demonstrated here (see Fig. 3) enables the Slo1 channel to respond to CO in a redox-independent manner. This mechanism contrasts with the redox- and heme-dependent mechanism suggested by an earlier study (36). Because the application of heme to the Slo1 BK channel decreases open probability at positive voltages (19, 20), it was suggested that CO activates the channel by relieving the inhibitory effect of reduced heme (36). The relative physiological relevance of the previously proposed redox- and heme-dependent mechanism (36) and the mechanism suggested by our study is not entirely clear. However, at negative and physiological voltages, heme increases open probability (20), and the reversal of its effect would be expected to inhibit the channel, rendering CO an inhibitor of the channel activity. Our results show that CO robustly increases open probability at such negative voltages in a redox-independent manner.

Slo1 BK channels are almost ubiquitously expressed in mammalian tissues and play pivotal roles in many physiological processes (6), including acute oxygen sensing (7, 37). Because Slo1 may colocalize with HMOX-2, which breaks down heme in an oxygen-dependent manner generating CO (1), regulation of the Slo1 BK channel activity by CO has been postulated as a mechanism of acute oxygen sensing (16). However, the results of the experiments using HMOX-2 null mice show that other hypoxia sensing mechanisms exist (38). Although the exact role of modulation of Slo1 BK channels by CO in acute oxygen sensing remains uncertain (38), it is clear that CO does activate Slo1 BK channels and contributes to many other physiological processes, including vasodilation (9, 11, 13–15). The ability of the Slo1 BK channel to directly respond to CO at physiological voltages at low to moderate $[\text{Ca}^{2+}]_i$ via the high-affinity RCK1 Ca^{2+} sensor further illustrates the channel's remarkable modulatory repertoire.

Materials and Methods

Channels. Slo1 BK channel complexes, human Slo1 (U11058) alone or Slo1 and $\beta 1$ (U38907) together, were expressed in HEK cells as described (39). Mutations were introduced using a commercially available PCR-based kit (Stratagene), and the sequences were verified (University of Pennsylvania DNA Sequencing Facility).

Electrophysiology and Data Analysis. Ionic currents were recorded in the inside-out configuration of the patch-clamp method and analyzed (20, 39). The extracellular solution typically contained 140 mM KCl, 2 mM MgCl_2 , and 10 mM Hepes (pH 7.2), with 3 mM *N*-methyl-D-glucamine (NMDG). The nominally Ca^{2+} -free internal solution contained 140 mM KCl, 11 mM EGTA (or EDTA), 10 mM Hepes (pH 7.2), with 25 mM NMDG. This solution is assumed to have ≤ 10 nM $[\text{Ca}^{2+}]$ as estimated by our earlier fura-2 based measurements (39). The solutions containing from 0.1 μM to 10 μM of Ca^{2+} were made with HEDTA, and those with greater $[\text{Ca}^{2+}]$ did not contain any chelator. Free divalent cation concentrations were calculated by Patcher's Power Tools assuming 20 μM of contaminating Ca^{2+} . The solutions with different $[\text{Mg}^{2+}]$ contained 11 mM EGTA and no added Ca^{2+} . Unless noted otherwise, leak and capacitive currents have been subtracted. The voltage dependence of channel activation was characterized by fitting the voltage dependence of

the extrapolated instantaneous tail current amplitude with a Boltzmann function so that each data set is described by the midpoint ($V_{0.5}$) and the steepness of the curve. Single-channel openings were analyzed as performed previously (39). Statistical comparisons were made by using Student's *t* test or ANOVA, followed by the Dunnett test where appropriate; the significance was assumed at $P \leq 0.05$. The results are presented as mean $\pm$ SEM (n = number of independent measurements).

Tricarbonyldichlororuthenium (II) dimer ($[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$) (Sigma) was freshly dissolved in DMSO to make 400 mM stock solution, and 1 μl of the stock solution was then quickly diluted into 8 ml of recording solution in a tightly sealed small vial to achieve the final concentration of 50 μM . This concentration is similar to those used in other studies (12) and represents a functionally saturating concentration on the Slo1 channel. The CO donor did not change the solution pH, and DMSO, at the final concentration (0.0125%), did not alter the Slo1 BK currents. All chemicals were freshly prepared before use. The CO released from the donor in each of the solutions used was verified spectrophotometrically (22). Briefly, the stock solution of the

CO donor (400 mM) was diluted to 50 mM by using a recording solution, and 1 μl of the diluted solution was quickly added into the recording solution containing myoglobin (100 μM) and 0.1% sodium dithionite. The absorbance value at 540 nm was measured as a function of time to estimate the kinetics of CO release. Such measurements confirmed that the CO donor quickly converted deoxy-Mb to MbCO (SI Fig. 11). CO gas was applied by using the method described previously by Farrugia *et al.* (41). The two CO application methods produced indistinguishable results (SI Fig. 5). Unless otherwise noted, the results presented were obtained by using the CO donor (50 μM) due to its ease of use. We verified that neither CO gas nor the donor altered $[\text{Ca}^{2+}]$ in the recording solutions by using fura-2. "Aged" CO donor solutions, the donor-containing solutions placed at room temperature for 7 days, had no effect on Slo1 BK channels (SI Fig. 6). Another recent study also implicates the Slo1 C-terminal domain in CO sensitivity (43).

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