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Mechanistic Insight into the Heme-independent Interplay between Iron and Carbon Monoxide in CFTR and *Slo1* BK_{Ca} Channels

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Abstract

Ion channels have been extensively reported as an effector of carbon monoxide (CO). However, the mechanisms of heme-independent CO action are still missing. Because most of ion channels are heterologously expressed on human embryonic kidney cells which are cultured in Fe³⁺-containing media, CO may act as a small and strong iron chelator to disrupt a putative iron bridge in ion channels and thus to tune their activity. In this review CFTR and *Slo1* BK_{Ca} channels are employed to discuss the possible heme-independent interplay between iron and CO. Our recent studies demonstrated a high-affinity Fe³⁺ site at the interface between the regulatory domain and intracellular loop 3 of CFTR. Because the binding of Fe³⁺ to CFTR prevents channel opening, the stimulatory effect of CO on the Cl⁻ and HCO₃⁻ currents across the apical membrane of rat distal colon may be due to the release of inhibitive Fe³⁺ by CO. In contrast, CO repeatedly stimulates the human *Slo1* BK_{Ca} channel opening possibly by binding to an unknown iron site because cyanide prohibits this heme-independent CO stimulation. Here, *in silico* research on recent structural data of the *slo1* BK_{Ca} channels indicates two putative binuclear Fe²⁺-binding motifs in the gating ring in which CO may compete with protein residues to bind to either Fe²⁺ bowl to disrupt the Fe²⁺ bridge but not to release Fe²⁺ from the channel. Thus, these two new regulation models of CO, iron releasing from and retaining in the ion channel, may have significant and extensive implications for other metalloproteins.

Keywords

ion channel; metalloprotein; gating; gas regulation; binuclear Fe bowl

Introduction

Carbon monoxide (CO) is emerging an intriguing role in actively regulating ion channels such as voltage-gated L-type Ca²⁺ (Ca_v) channels^{1–2}, background tandem P domain K⁺ (K_{2p}) channels³, large-conductance, voltage- and Ca²⁺-activated K⁺ (BK_{Ca}) channels⁴, epithelial Na⁺ channels (ENaC)^{5–6}, P2X receptor⁷, voltage-gated K⁺ (K_v) channels⁸, voltage-gated sodium (Na_v 1) channels⁹, and T-type Ca²⁺ channels¹⁰. These ion channels are

Conflict of interest

None.

generally heterologously expressed on human embryonic kidney (*HEK*) cells. Their exposure to a CO-releasing molecule (CORM-2), tricarbonyl dichlororuthenium (II) dimer ($[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$), stimulates or inhibits channel activity^{11–12}. Because the *HEK* cells are cultured in Fe^{3+} -containing media such as Dulbecco's modified Eagle's medium (DMEM) and there are some trace amount of Fe^{2+} or Fe^{3+} in cells under physiological conditions, Fe^{2+} or Fe^{3+} may be involved in these events. However, most of experiments were done by using the whole-cell configuration, CO may act as a signal molecule (cellular messenger) to modulate channel activity via several intracellular signal pathways. For example, the products of heme catalyzed by heme oxygenase (HO) have not only CO but also Fe^{2+} and biliverdin. Which product specifically acts on ion channels are unknown. In addition, CORM-2 may also directly regulate channel activity and have nothing to do with the CO gas. Until now only *Slo1* BK_{Ca} channels were studied at detail by using the inside-out membrane patch and the CO gas. Because repeated activation of this *Slo1* channel by CO is prohibited not by the removal of heme-binding site but by cyanide^{4, 13}, and the non-bonding lone pair of electrons in carbon can render strong transition metal binding to CO, it is possible that CO may bind to a putative Fe site but not to release the Fe ion from the channel. On the other hand, CORM-2 has been reported to evoke the Cl^- and HCO_3^- currents across the apical membrane of rat distal colon¹⁴, CO and the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel in colon may be involved. Because our recent studies indicated an inhibitive Fe^{3+} site with a high affinity for CFTR^{15–16}, CO may stimulate the CFTR activity by chelating and releasing Fe^{3+} from the CFTR. Thus, these two case studies on the potential heme-independent interplay between iron and CO in BK_{Ca} and CFTR channels may reveal two new regulation modes of CO which will prompt mechanistic understanding of heme-independent action of CO on other ion channels and metalloproteins.

CFTR channel

CFTR, a unique and important member of ATP binding cassette (ABC) superfamily, serves as not an ATP-driven pump but an ATP-gated anion channel. It regulates fluid and electrolyte across epithelial surface and resultant mucociliary clearance in multiple organs including lung, pancreas, liver, sweat duct, intestines and reproductive tract. Genetic mutation-induced dysfunction of human CFTR at the respiratory epithelium causes cystic fibrosis (CF), the most common monogenic inherited disorder among *Caucasians*. This genetic disease brings about many adult CF patients to be chronically infected with *Pseudomonas aeruginosa* (*Pa*) in the CF lung so that resultant biofilm formation enhances antibiotic resistance of *Pa* by acquiring iron. For example, the most common CF-causing mutation F508del increases iron release by human airway epithelial cells and thus promotes the exuberant formation of antibiotic-resistant biofilms while Fe^{3+} chelation with deferoxamine or deferasirox or hypothiocyanite prevents *Pa* biofilm formation and eliminates established biofilms on CF airway cells overexpressing F508del^{17–19}. Because Corr-4a increases F508del-CFTR-mediated chloride secretion in the plasma membrane and reduces biofilm formation without changing the iron concentration in the apical medium²⁰, limiting iron acquisition and increasing CFTR activity on airways may be an attractive therapeutic strategy to reduce *Pa* biofilm formation.

multitasking role in activating cytoprotective pathway and CFTR expression in airway epithelial cells^{45–46}, increasing intracellular CO levels could be a promising therapy against lung infection and inflammation in patients with CF.

On the other hand, as the inhibitive Fe^{3+} site in CFTR has been well defined after the channel is expressed in *HEK* cells which are cultured in the Fe^{3+} -containing medium, an Fe^{2+} or Fe^{3+} site may also be involved in other CO-sensitive ion channels such as *Slo1* BK_{Ca} when expressed in an $\text{Fe}^{2+}/\text{Fe}^{3+}$ -containing physiological environment.

***Slo1* BK_{Ca} channel**

The large-conductance voltage- and calcium-activated human *Slo1* BK_{Ca} channel is a critical member of the high-conductance *Slo* family of K^+ channel^{47–49}. A coupling of electrical signaling to Ca^{2+} -mediated events allows this channel to regulate muscle contraction, hormone secretion and neuronal excitability^{50–51}. Recent $\text{Mg}^{2+}/\text{Ca}^{2+}$ -free and $\text{Mg}^{2+}/\text{Ca}^{2+}$ -bound cryo-EM structures of the full-length *Slo1* channel from *Aplysia californica* demonstrated that the BK_{Ca} channel is a tetramer with the transmembrane domain (TMD) on top, the first regulator of K^+ conductance (RCK1) domain in the middle and the RCK2 domain at the bottom (Figure 2)^{52–53}. The TMD includes S0 helix, a voltage-sensor domain (VSD) with S1–S4 helices and a pore domain (PD) with S5–S6 helices. Because the VSD of one subunit does not interact with the PD of the other neighboring subunit but the TMD of one subunit interacts with the RCK1 of the other neighboring subunit, the VSD is not domain-swapped with the PD while the TMD is swapped with the RCK1. The homology model of the human *Slo1* BK_{Ca} channel based on these two structures reveals two Ca^{2+} -binding sites and an Mg^{2+} -binding site in the RCK1 and RCK2 domains which form a gating ring. The Ca^{2+} site consists of D367, R514, G533, E535 and E604 in the RCK1 domain, the Ca^{2+} bowl site is composed of Q889, D892, D895, D897 from one RCK2 domain and N449 from the other neighboring RCK1 domain, and the Mg^{2+} site comprises of N172, E339, E374, T396, E399 between the VSD and the RCK1 domain. The Ca^{2+} bowl has the strongest affinity for Ca^{2+} while the Mg^{2+} site has the weakest affinity⁵⁴. Because R514 in the RCK1 domain forms an ionized H-bond with E902 and a cation- π interaction with Y904 in the RCK2 domain, the two Ca^{2+} sites may cooperatively interact with each other to activate the *Slo1* BK_{Ca} channel. In addition, the RCK1 N-lobe, which is shared by both Ca^{2+} binding sites via N449 or D367, tethers the C terminus of S6 via a polypeptide chain linkage or specifically interfaces with the voltage sensor/S4–S5 linker. Therefore, a rigid body titling of the RCK1 N-lobe may directly or indirectly drive a movement of S6 for channel opening in response to Ca^{2+} binding to the RCK1 or the RCK2 while the movement of S4 helices in VSD can in turn exert an effect on the gating ring and the apparent Ca^{2+} binding affinity^{52–53}. Recent electrophysiological measurements also support the notion that the Ca^{2+} -sensitive gating ring facilitates the effective coupling between the VSD and the PD⁵⁵.

The *Slo1* BK_{Ca} channel has a conserved heme-binding sequence motif ⁶¹²CXXCH⁶¹⁶ in the RCK1-RCK2 linker⁵⁶. The binding of both heme and hemin to H616, the axial ligand of this human *Slo1* channel, increases P_o at negative voltages but decreases P_o at more positive voltages possibly by disrupting both Ca^{2+} - and voltage-dependent gating via the gating

ring^{57–59}. Further studies defined heme-sensitive C612 and C615 as a thiol/disulfide redox switch which controls its affinity for heme and CO and HO-2 and thus tunes channel gating in response to changes in the redox state of the cell under hypoxic and normoxic conditions⁶⁰. However, Hou and coworkers found that, in the absence of Mg^{2+} and Ca^{2+} , the channel opening of *Slo1* BK_{Ca} by CO is inhibited not by the heme-insensitive mutation H616R or C615S/H616R or internal H_2O_2 or acidification pretreatment but by histidine modifier diethyl pyrocarbonate (DEPC) or the heme-sensitive mutation H365R or H394R or H365R/H394R or H365A/H394A or D367A in the RCK1 domain. They further observed that the CO sensitivity is prohibited not by Mg^{2+} -insensitive mutation E374A or E399A but by intracellular Ca^{2+} at the saturating concentration (120 μM)⁴. Thus, the heme-independent CO stimulation found in the Ca^{2+} -free environment is Ca^{2+} -independent.

On the other hand, Kemp and coworkers reported a different case. Although activation of the *Slo1* BK_{Ca} channel by CO is partially dependent on $[Ca^{2+}]_i$ in the physiological range (0.1–1 μM), in the presence of 2 mM Mg^{2+} and 300 nM Ca^{2+} it is not completely prohibited by single or double mutation of H365 and H394 but by 5 mM EGTA or saturating $[Ca^{2+}]_i$ (>10 μM) or cyanide or the replacement of the Ca^{2+} -sensitive S9–S10 module of the C-terminal of *hSlo1* with the Ca^{2+} -insensitive S9–S10 module of the C-terminal of *mSlo1*^{61–62}. They also reported that the mutant H365R/H394R in RCK1 domain actually enhances CO sensitivity when $[Ca^{2+}]_i$ is in the physiological range. Although CO still potentiates the activity of the heme-bound *Slo1* BK_{Ca} channel at a higher Ca^{2+} concentration (10 μM) by binding to heme^{59–61}, CO actually stimulates the hemin-insensitive H616R mutant in the presence of 336 nM Ca^{2+} ⁶². Because of the rapid reversibility of CO and cyanide binding, they proposed that CO activates the *Slo1* BK_{Ca} channel by binding to a transition metal cluster⁶². As all these experiments were carried out by using *HEK*-293 cells which were cultured in the Fe^{3+} -containing medium, and C911G in the RCK2 partially weakens the CO sensitivity⁶², it is possible that C911 may dynamically bind to an Fe site for channel closure and CO may open the BK_{Ca} channel by competing off C911 from the Fe site. Supporting this dynamic binding of C911 to the Fe site, Tang and collaborators found that C911 can be oxidized by H_2O_2 or MTSEA⁶³. This case is similar to the Zn^{2+} -dependent redox switch at the intracellular dynamic T1-T1 interface of a Kv4.1 channel in which Zn^{2+} is tightly bound to H104, C131 and C132 in one T1 domain but weakly bound to C110 in the other neighboring T1 domain⁶⁴. It is interesting that modification of C911 by H_2O_2 or MTSEA still inhibits the BK_{Ca} channel current⁶³. Thus, oxidation C911 by H_2O_2 or MTSEA may still confer H-bonds to the Fe ligands. More importantly, because the CO effect was still observed in the presence of 11 mM metal chelator EGTA or EDTA and repeated after a washout⁴, Fe ions may not be released from the high-affinity site by EGTA or EDTA or CN^- or CO. In other words, the binding stability constant ($\log K$) of Fe ions for the *Slo1* BK1 channel may be more than 35 because $\log K$ of Fe(II) and Fe(III) for cyanide are 35 and 42, respectively and $\log K$ of Fe(II) and Fe(III) for EGTA are 11.8 and 20, respectively^{65–66}. These results are different from those observed in the Kv4.1 channel in which the disulfide bond between C132 and C110 or the H-bond between H104 and oxidized C110 can be formed unless Zn^{2+} is removed from C131, C132 and H104⁶⁴. Thus, the putative abnormal heme-independent Fe site in the *Slo1* BK_{Ca} channel may not be coordinated by canonical protein residues such as cysteine, aspartate or glutamate as found in CFTR but by

hydrophobic aromatic residues which may protect Fe ions from releasing by acidification or chelators such as EDTA, EGTA, CO and CN^- .

Sequence alignment of *Slo* BK channels demonstrates a conserved motif $^{391}\text{F}_{\text{XXH}}\text{F}^{394}$ and a highly-conserved motif $^{524}\text{WXXX}\text{Y}/\text{F}^{528}$ across the species (Figure 3), which may function like water-insoluble *cis*-cyclopentadienyl iron (II) dicarbonyl dimer to form a binuclear Fe^{2+} bowl to interact with CO or CN^- . Recent structural models of the *Slo1* BK_{Ca} channels showed that a conserved motif $^{365}\text{HXDR}^{368}$ is 8 Å distant from the former Fe^{2+} -binding motif and 4.5 Å away from R514/E902/Y904 near the Ca^{2+} RCK1 site while a conserved motif $^{907}\text{QXXX}\text{C}^{911}$ is 6 Å distant from the latter Fe^{2+} -binding motif and 3.5 Å away from the Ca^{2+} bowl^{54–55, 67–68}. Thus, two Fe^{2+} can be sandwiched by F391 and H394 via cation- π interactions to form the first putative binuclear Fe^{2+} bowl to which H365/D367 or CO or CN^- can bind regulating channel gating via the non-swapping interactions of R514 with E902 and Y904 (Figure 4). In the meanwhile, another pair of Fe^{2+} can be sandwiched by W524 and Y528 via cation- π interactions to produce a second binuclear Fe^{2+} bowl to which Q907/C911 or CO or CN^- can bind modulating channel gating via the swapping interactions of N449 with the Ca^{2+} bowl (Figure 5).

In the absence of Ca^{2+} and Mg^{2+} , N449 and the Ca^{2+} bowl may be disconnected, the binding of either Q907/C911 or CO or CN^- to the second Fe^{2+} bowl would not alter channel activity. However, the binding of H365/D367 to the first Fe^{2+} bowl may facilitate channel closure possibly by disrupting the non-swapping interactions of R514 with E902 and Y904. CO may compete off H365/D367 from the first Fe^{2+} bowl to promote channel opening by rebuilding the non-swapping interactions of R514 with E902 and Y904⁴. In this case, CN^- is expected to reverse the CO stimulation possibly by forming a salt bridge with R368 (Figure 4). The mutation of H365A/R or H394A/R or D367A/R may disrupt the first inhibitive Fe^{2+} bridge and thus fail to evoke the CO effect⁴. A mutation of F391A may also be expected to render the channel insensitive to CO in the absence of Ca^{2+} . However, the mutation of Q907A/C911A may not suppress the Ca^{2+} -independent CO stimulation. In addition, acidic activation of the human *Slo1* BK_{Ca} channel by protonation of H365, D367 or H394 may disrupt the first inhibitive Fe^{2+} bridge⁶⁹. Finally, activation of *Slo1* BK_{Ca} channel by Zn^{2+} binding to H365/D367/E399 may also result partly from the disconnection of the first inhibitive Fe^{2+} bridge⁷⁰.

In the presence of 2 mM Mg^{2+} and 5 mM EGTA⁶², the free Mg^{2+} concentration is about 40 μM ($\log K$ of Mg^{2+} for EGTA is about 5⁶⁶). Because Mg^{2+} is coordinated with six oxygen atoms⁷¹, the binding of Mg^{2+} to the Ca^{2+} bowl may leave N449 away from the Ca^{2+} bowl⁷². In the meanwhile, the Mg^{2+} -bound Ca^{2+} bowl may also disrupt the interactions of R514 with E902 and Y904 possibly as a result of a cooperation between two Ca^{2+} sites^{52–53}. In this case, the release of H365/D367 from the first Fe^{2+} bowl and the separation of Q907/C911 from the second Fe^{2+} bowl by CO or CN^- would not modulate channel gating (Figure 5)⁶².

At a low Ca^{2+} concentration ($<0.1 \mu\text{M}$), the binding of Ca^{2+} to the Ca^{2+} bowl may break up both the swapping interaction of N449 with the Ca^{2+} bowl and the non-swapping interactions of R513 with E902 and Y904 so that the channel is irresponsive to CO or

CN⁻⁶¹. In the physiological range of Ca²⁺ (0.1–1 μ M), even if the mutation of H365R/H394R damages the first Fe²⁺ bridge, CO may still release Q907 and C911 from the second Fe²⁺ bowl and promote Ca²⁺ bridging N449 with the Ca²⁺ bowl for channel opening while CN⁻ may reverse the CO stimulation possibly by forming an inhibitive salt bridge with K1030 and thus disrupting the Ca²⁺ bridge of N449 with the Ca²⁺ bowl (Figure 5)⁶². When C911 is modified by H O 63 2 2 or MTSEA, the introduced adduct may form a putative H-bond with the Fe²⁺ ligand Y527 or Y528 to prevent the stimulatory Ca²⁺ bridge between N449 and the Ca²⁺ bowl. When the RCK2 domain of the human *Slo1* channel is replaced with that of *mSlo3*, this chimeric channel is not activated by Ca²⁺⁶¹. Therefore, even if Ca²⁺ binds to E880 and E886 at the Ca²⁺ bowl, N449 is separated from the Ca²⁺ bowl while R514 may not interact with S893 and H895 possibly as a result of RCK1-RCK2 cooperation^{52–53}. What is more, Q907/C911 have been substituted by T899/T903 in this chimeric channel and thus the second Fe²⁺ bridge is broken (Figure 3). In this case, even if CO and CN⁻ can compete off H365/D367 from the first Fe²⁺ bowl, they may not regulate channel gating in either Ca²⁺ independent or Ca²⁺-independent manner. At a saturation Ca²⁺ concentration (>10 μ M), the strong binding of Ca²⁺ to the Ca²⁺ bowl and the Ca²⁺ RCK1 site may disconnect both inhibitive Fe²⁺ bridges and thus the binding of CO or CN⁻ to either Fe²⁺ bowl would no longer change channel gating^{4,61}. However, CO can still drive channel opening by binding to heme once bound to *Slo1* BK_{Ca}⁵⁹.

Miranda and coworkers used patch-clamp fluorometry and observed the 30° rotation of RCK2 against RCK1 with channel activation⁷³, which is not shown in recent structural models of the BK channel^{52–53, 67–68}. Thus, the Ca²⁺/Mg²⁺-free *Slo1* BK channel may Ca not be fully closed possibly as a result of the absence of two inhibitive Fe²⁺ bridges⁵³. In the presence of 10 mM Mg²⁺ or 200 mM Ba²⁺, the binding of Mg²⁺ or Ba²⁺ to the Ca²⁺ bowl, as required by six coordination oxygen atoms⁷¹, may expand the Ca²⁺ bowl and shrink the RCK1-RCK2 linker but may not disrupt two Fe²⁺ bridges⁷². In the presence of 100 μ M Cd²⁺, the possible binding of Cd²⁺ to H365 and D367 and E399, as Zn²⁺ does⁶⁸, may only shrink the RCK1-RCK2 linker and disrupt the first Fe²⁺ bridge⁷¹. However, the binding of Ca²⁺ to the Ca²⁺ bowl and the Ca²⁺ RCK1 site may not only disrupt two Fe²⁺ bridges but also enhance the swapping interactions of N449 with the Ca²⁺ bowl and the non-swapping interactions of R514 with E902/Y904 so that the gating ring may be dramatically rearranged (Figure 6)^{52–53,73}. In other words, the Fe²⁺ bowl may contribute to the large changes in the FRET signal directly or indirectly^{72–73}. In this regard, CO is expected to induce a large conformation change in the gating ring and CN⁻ can reverse it (Figure 6).

Taken together, the first putative Fe²⁺ bridge may be coupled to the non-swapping interactions of R514 with E902 and Y904 while the second putative Fe²⁺ bridge may be coupled to the swapping interactions of N449 with the Ca²⁺ bowl. Although the disruption of the first and second Fe²⁺ bridges by CO may stimulate channel opening in both Ca²⁺-independent and Ca²⁺-dependent manners, respectively, both non-swapping and swapping interactions may facilitate a positive RCK1-RCK2 cooperation (Figure 6). In addition to the stimulatory swapping Mg²⁺ binding sites at the TMD-RCK1 interface, the stimulatory Ca²⁺ RCK1 site, the stimulatory swapping Ca²⁺ bridge at the RCK1-RCK2 interface and the inhibitory heme site at the RCK1-RCK2 linker, two non-swapping inhibitory Fe²⁺ sites, one at the RCK1-RCK2 interface and the other in the RCK1 domain, are to be identified for full

channel closure and a large movement of the gating ring and gating regulation by CO and CN^- .

It is also exciting to examine the heme-independent interplay between Fe^{2+} and CO in other *Slo* channels. Figure 3 suggests that most of the *Slo1* BK_{Ca} channels have a potential of the Ca^{2+} -dependent or independent CO stimulation even if the heme site is removed because they have not only two highly-conserved Fe^{2+} binding motifs but also two kinds of highly-conserved RCK1-RCK2 interactions. Since the *hSlo3* BK_{Ca} channel is activated by Ca^{2+} but the *mSlo3* BK_{Ca} channel is not⁷⁴, *hSlo3* is expected to retain the first Fe^{2+} binding site and the Ca^{2+} RCK1 site and the swapping interactions of N438 with N894 and H896 so that the heme- and Ca^{2+} -independent CO stimulation is expected (Figure 3). However, both *hSlo3* and *mSlo3* BK_{Ca} channels may exhibit a heme-dependent CO stimulation because they maintain the heme-binding motif⁷⁴. For *Slo2* channels, although they may have the first Fe^{2+} bridging motif, the non-swapping RCK1-RCK2 interactions are absent. On the other hand, although they may have a second Fe^{2+} bowl, no residue bridges with it. What is more, they have not a heme binding motif⁷⁵. Accordingly, no CO stimulation would be observed.

Conclusions

Heme-independent activation of the CFTR channel and the *Slo1* BK_{Ca} channel by CO may represent two different modes of gating regulation of ion channels by CO. In the case of CFTR, Fe^{3+} is bound to the R-ICL3 interface^{15–16}. This interfacial Fe^{3+} site is formed by traditional coordination ligands such as H950 and H954 from ICL3 and C832 and D836 from the R domain (Figure 1). This Fe^{3+} binding motif may be 832CXXXDXnHXXXH954. Once Fe^{3+} is released by CO, a washout cannot reverse the channel activity so that the CO effect would not be repeated. In the case of the human *Slo1* BK_{Ca} channel, Fe^{2+} may be always located in a bowl with a putative motif of 524WXXYY/F⁵²⁸ or 391FXXHF395 no matter whether the channel is opened or closed by a change in voltage or Ca^{2+} or Mg^{2+} concentrations. However, the dynamic binding of H365/D367 to the first Fe^{2+} bowl or the dynamic binding of Q907/C911 to the second one may promote channel closure. Thus, CO may compete off H365/D367 and Q907/C911 from the first and second Fe^{2+} bowls for channel opening in Ca^{2+} -independent and Ca^{2+} -dependent manners, respectively (Figure 4–6). In this case, a washout followed by the CO stimulation can reverse the channel activity and thereby the CO effect can be repeated. Which regulation mode of heme-independent CO action works in ion channels may depend on whether the CO effect can be repeated or not upon a washout. More experiments by using inside-out membrane patches of *HEK* cells expressing ion channels may further define these two modes and enrich the interplay between iron and CO in ion channels. In the case of heme-dependent CO regulation of ion channels, if heme can be washed out from ion channels such as ENaC^{76} , the potential CO effect on channel activity cannot be repeated. However, if heme cannot be washed out from ion channels such as *Slo1* BK^{57} Ca , the possible CO effect on channel activity may still be repeated once heme is bound. Therefore, the heme-independent regulation of ion channels by CO should be defined without a heme binding site. Given that both apical CFTR and *Slo1* BK_{Ca} channels in airway epithelial cells play a critical role in airway hydration and volume homeostasis^{77–79}, their CO stimulation may protect CF patients against lung infection and inflammation. Since CO may target other metalloproteins to which transition metals are

bound, these two heme-independent regulation modes of CO may have significant and extensive implications for those metalloproteins.

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Abbreviations

ABC	ATP-binding cassette
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CO	carbon monoxide
CORM	CO-releasing molecule
DMEM	Dulbecco's modified Eagle's medium
EM	electron microscopy
ENaC	epithelial Na ⁺ channel
HO	heme oxygenase
FDA	Food and Drug Administration
FRET	fluorescence resonance energy transfer
HEK	Human embryonic kidney
DEPC	histidine modifier diethyl pyrocarbonate
ICL	intracellular loop
MTSEA	2-aminoethyl methanethiosulfonate hydrobromide
NPPB	5-nitro-2-(3-phenylpropylamino)benzoate
NPPB-AM	5-nitro-2-(3-phenylpropylamino)benzamide
NBD	nucleotide binding domain
PCAT	peptidase-containing ATP-binding cassette transporter
PD	pore domain
PKA	protein kinase A
<i>Pa</i>	<i>pseudomonas aeruginosa</i>
R	regulatory

RCK	regulator of K ⁺ conductance
K_{2p}	tandem P domain K ⁺
TMD	transmembrane domain
VSD	voltage-sensor domain

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Figure 1. The tentative interplay between Fe³⁺ and CO in the human CFTR channel
 Fe³⁺ prevents channel opening by bridging the C-terminal part of the R domain (R2) with intracellular loop 3 (ICL3) and allowing the N-terminal part of the R domain (R1) to block the conductance pathway and to prevent the ATP binding-induced NBD1-NBD2 dimerization. In the non-phosphorylated state, the Fe³⁺ ligands include C832 and D836 in the R2 domain and H954 in ICL3 while the PKA site S768 (not shown) in the R2 domain H-bonds with H950 from ICL3. Upon phosphorylation of the R domain by PKA, phosphorylated S768 (not shown) and H775 (not shown) in the R2 domain and H950 are also bound to Fe³⁺ at the R2-ICL3 interface^{15–16}. Once Fe³⁺ is removed by CO, the

phosphorylated R domain can bind to the ICL1/ICL4 interface via a putative cation- π interaction of the R/K in a PKA site of the R domain with Y161 and F1078 for channel opening by ATP (not shown)³¹. (A), The closed and open states were prepared with on the cryo-electron microscopy structure of human CFTR and the crystal structures of bacterial transporters *Sav1866*, respectively^{22, 24}. The individual domains or residues are colored uniquely: cyan, TMD1 and NBD1; light gray, TMD2 and NBD2; red, Lasso motif; blue, unphosphorylated R1 and R2 domains; red, phosphorylated R1, R2 or R domain; forest green, Y161 and F1078; magenta, H950 and H954; orange, Fe³⁺. (B), The closed and open states were based on the cryo-electron microscopy structure of human CFTR and the crystal structure of the peptidase-containing ATP-binding cassette transporter (PCAT) 1 from *Clostridium thermocellum*, respectively^{24, 33}.

Figure 3. Sequence alignment of *Slo* family members

Residues discussed in the text are bolded and colored. Numbers above and below the sequences refer to the human *Slo1* and the chicken *Slo2.2*, respectively⁷⁴⁻⁷⁵. For the human *Slo1* BK_{Ca} channel, both the non-swapping interactions of R514 with E902 and Y904 and the swapping interactions of N449 with Q889, D892, D895 and D897 may facilitate a positive RCK1-RCK2 cooperation and channel opening⁵²⁻⁵³. The former may be coupled to the first putative Fe²⁺ bridge between H365/D367 and the first putative Fe²⁺ bowl formed by F391/H394 while the latter may be coupled to the second putative Fe²⁺ bridge between Q907/C911 and the second putative Fe²⁺ bowl formed by W524/Y528. CO may target two putative Fe²⁺ bowls to disrupt two inhibitive Fe²⁺ bridges while CN⁻ may compete off CO and form a prohibitive salt bridge with counterion R368 or K1030.

Figure 4. The tentative heme- and Ca^{2+} -independent CO stimulation pathway of the human *Slo1* BK_{Ca} channel

The *in silico* models were based on the crystal structure of the *Slo1* BK_{Ca} channel from *Aplysia californica*⁵³. F391 and H394 in the RCK1 may form a high-affinity binuclear Fe^{2+} bowl via cation- π interactions to prevent a small or large iron chelator to access and to release iron ions. The binding of H365 and D367 to the Fe^{2+} bowl may disrupt the electrostatic interaction of R514 with E902 and the cation- π interaction of R514 with Y904 and thus promote channel closure. However, CO can compete off H365 and D367 from the Fe^{2+} bowl and thus reestablish the non-swapping interactions of R514 with E902 and Y904 for channel opening. CN^- may compete off CO from the Fe^{2+} bowl and form an electrostatic attraction with R368 and thus enhance channel closure by disrupting the nonswapping interactions of R514 with E902 and Y904. In any case, the binding of Ca^{2+} to D367 and R514, G533, E535 and E604 would alleviate the CO or CN^- effect by weakening the Fe^{2+} bridge between H365/D367 and F391/H394.

Figure 5. The tentative heme-independent but Ca^{2+} -dependent CO stimulation pathway of the human *Slo1* BK Ca channel

The *in silico* models were based on the crystal structure of the gating ring of the human *Slo1* BK Ca channel⁵². W524, Q525, Y527 and Y528 in the RCK1 may form a high-affinity binuclear Fe^{2+} bowl to prevent a small or large iron chelator to access and to release iron ions. Two Fe^{2+} may be sandwiched by W524 and Y528 via cation- π interactions while Q525 may render an additional coordination with two Fe^{2+} ions to hold the metal in the bowl. Y527 may protect Fe^{2+} from access of a large chelator. At a lower Ca^{2+} concentration ($<10\ \mu\text{M}$), the binding of Q907 and C911 in the RCK2 domain to the second Fe^{2+} bowl in RCK1 domain may disrupt the swapping Ca^{2+} bridge between N449 and the Ca^{2+} bowl and thus promote channel closure. CO may bind to Fe^{2+} to release Q907 and C911 from the second Fe^{2+} bowl and thus to reestablish the intersubunit Ca^{2+} bridge for channel opening. CN^- may compete off CO from the second Fe^{2+} bowl and form an electrostatic attraction with K1030 in the RCK2 domain. The resultant disruption of the swapping RCK1-RCK2 Ca^{2+} bridge may promote channel closure. However, at a higher Ca^{2+} concentration ($>10\ \mu\text{M}$), Ca^{2+} bridging N449 with the Ca^{2+} bowl may pull Q907 and C911 or K1030 away from the second Fe bowl so that no CO or CN^- effect would be observed. On the other hand, because Mg^{2+} is coordinated by 6 oxygen atoms⁷¹, the binding of Mg^{2+} to the Ca^{2+} bowl may disrupt both the swapping interaction between N449 and the Ca^{2+} bowl and the non-

swapping interactions of R514 with E902/Y904 as a result of the RCK1-RCK2 cooperation^{52–53, 72}. In this case, neither CO nor CN[−] can regulate channel activity.

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Figure 6. The tentative heme-independent gating regulation of the human *Slo1* BK_{Ca} channel by Ca²⁺ or CO or CN⁻

The *in silico* models were based on the crystal structures of the gating ring of the human *Slo1* BK_{Ca} channel^{52–53}. Two intracellular tandem C-terminal RCK1 and RCK2 from each of four channel subunits form a gating ring. Eight pairs of Fe²⁺ ions may always be bound to the channel. Either the swapping interactions between N449 and the Ca²⁺ bowl or the non-swapping interactions between R513 and E902/Y904 near the Ca²⁺ bowl may be required for channel opening^{52–53}. In the absence of Ca²⁺, the swapping bridges between N449 and the Ca²⁺ bowl are broken. The Fe²⁺ bridge between H365/D367 and F391/H394 in RCK1 may also disrupt the non-swapping interactions of R514 with E902 and Y904 so that the channel is fully closed. CO may disrupt this first Fe²⁺ bridge at the Ca²⁺ RCK1 site and thus enhance channel opening while CN⁻ can reverse the CO effect. When Ca²⁺ (<10 μM) is added, the Ca²⁺ bridge between N449 and the Ca²⁺ bowl may further promote channel opening because CO has disrupted the second Fe²⁺ bridge between Q907/C911 and W524/Y528 near the Ca²⁺ bowl. On the other hand, if Ca²⁺ (<10 μM) is first applied, the binding of Ca²⁺ to N449 at the Ca²⁺ bowl or D367 at the Ca²⁺ RCK1 site may weaken both Fe²⁺ bridges for the stimulatory interactions between N449 and the Ca²⁺ bowl and between R514 and E902/Y904. CO may completely disrupt both Fe²⁺ bridges so as to promote maximal channel opening. At saturation concentration of Ca²⁺ (>10 μM), once Ca²⁺ bridges N449 with the Ca²⁺ bowl, the binding of Ca²⁺ to the Ca²⁺ RCK1 site may drive a gating rotation

of RCK1 against RCK2 and TMD. This rotation may expand both RCK1 and RCK2 but shrink the RCK1-RCK2 linker and finally turn TMDs for maximal channel opening⁷³.

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