Heme Is a Carbon Monoxide Receptor for Large-Conductance Ca\(^{2+}\)-Activated K\(^{+}\) Channels

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Abstract—Carbon monoxide (CO) is an endogenous paracrine and autocrine gaseous messenger that regulates physiological functions in a wide variety of tissues. CO induces vasodilation by activating arterial smooth muscle large-conductance Ca\(^{2+}\)-activated potassium (BK\(_{Ca}\)) channels. However, the mechanism by which CO activates BK\(_{Ca}\) channels remains unclear. Here, we tested the hypothesis that CO activates BK\(_{Ca}\) channels by binding to channel-bound heme, a BK\(_{Ca}\) channel inhibitor, and altering the interaction between heme and the conserved heme-binding domain (HBD) of the channel \(\alpha\) subunit C terminus. Data obtained using thin-layer chromatography, spectrophotometry, mass spectrometry (MS), and MS-MS indicate that CO modifies the binding of reduced heme to the \(\alpha\) subunit HBD. In contrast, CO does not alter the interaction between the HBD and oxidized heme (hemin), to which CO cannot bind. Consistent with these findings, electrophysiological measurements of native and cloned (cbv) cerebral artery smooth muscle BK\(_{Ca}\) channels show that CO reverses BK\(_{Ca}\) channel inhibition by heme but not by hemin. Site-directed mutagenesis of the cbv HBD from CKACH to CKASR abolished both heme-induced channel inhibition and CO-induced activation. Furthermore, on binding CO, heme switches from being a channel inhibitor to an activator. These findings indicate that reduced heme is a functional CO receptor for BK\(_{Ca}\) channels, introduce a unique mechanism by which CO regulates the activity of a target protein, and reveal a novel process by which a gaseous messenger regulates ion channel activity. (Circ Res. 2005;97:805-812.)

Key Words: vascular smooth muscle ■ vasodilation ■ potassium channels ■ signal transduction

Large-conductance Ca\(^{2+}\)-activated potassium (BK\(_{Ca}\)) channels regulate the physiological functions of many tissues, including smooth muscle, neuronal and endocrine cells. BK\(_{Ca}\) channels are typically composed of pore-forming \(\alpha\) subunits that are encoded by the Slo1 (or KCNMA1) gene, and accessory \(\beta\) subunits that modulate channel gating. In smooth muscle cells, BK\(_{Ca}\) channels regulate cellular membrane potential and, thus, Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels, providing a mechanism to control contractility.

BK\(_{Ca}\) channel activity is regulated by a variety of signaling molecules, including intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)), protein kinases, tyrosine kinases, cytochrome P-450 metabolites of arachidonic acid, and heme. BK\(_{Ca}\) channels are also activated by physiologically relevant gases, including O\(_2\), CO, and NO. Although these gases can use cellular signaling pathways, O\(_2\), CO, and NO also activate BK\(_{Ca}\) channels in cell-free membrane patches isolated from the intracellular milieu.

Carbon monoxide is a physiological paracrine and autocrine messenger and neurotransmitter produced by heme oxygenase (HO) catalyzed metabolism of heme. Heme is found in virtually all cell types, and many cell types contain HO-2, including arterial smooth muscle cells, endothelial cells, and neurons. CO regulates a variety of physiological processes, including growth, smooth muscle contractility, neuronal excitability, and apoptosis. CO generated in both endothelial and smooth muscle cells induces vasodilation by activating BK\(_{Ca}\) channels. Recently, O\(_2\) has been reported to stimulate BK\(_{Ca}\) channels by acting as a substrate for HO-2, leading to the generation of CO, the downstream channel activator. Because CO activates arterial smooth muscle and carotid body BK\(_{Ca}\) channels in excised membrane patches, CO may bind directly to the channel protein itself or to a tightly associated regulatory molecule. However, how relatively inert CO interacts with and activates BK\(_{Ca}\) channels is unclear.

Free heme regulates human BK\(_{Ca}\) channel activity by binding with high affinity to a conserved amino acid sequence (CXXCH, where X is any amino acid) that is located between the 2 regulator of conductance for K\(^{+}\) (RCK) domains present in the \(\alpha\) subunit. A recognized property of...
reduced heme, particularly when contained within heme proteins, is its ability to bind diatomic gases, including CO. Thus, we tested the hypothesis that CO activates BKCa channels by binding to heme and modifying its interaction with the α subunit heme-binding domain.

Materials and Methods

Thin-Layer Chromatography

Native heme-binding pocket peptide (DAKEVKKAFYYKACHD-DITDPK, BKPP) and double mutant (C to S and H to R) peptide (DAKEVKKAFYYKASRSDITDPK; BKMP) were dissolved in water (stock solution: 1 mmol/L). Peptides (10 μL) were applied 2 cm from the bottom edge of 5×20 cm silica gel 60 plates and allowed to dry. Deoxygenated Krebs (dKrebs) was prepared by vigorously diffusing N2 through Krebs solution in a parafilm-sealed Erlenmeyer flask. Fe2+ heme solution was prepared by dissolving FeCl3·6H2O (0.5 mmol/L SDT and anoxic Krebs), CO (1 mmol/L) was added to make a final concentration of 20 to 40 μmol/L peptide or protein solutions. For MS, collision energies of 5 or 8 V were used. The voltages were kept very low to enable peak visualization of weakly bound ion complexes. Spray needle voltage was 1.8 kV, and cone voltage was 5V. Solutions were sprayed in 25 mmol/L ammonium bicarbonate, 12% to 25% acetonitrile. In all cases, stable native protein or peptide charge states were formed. For MS-MS, progressively increasing voltages were used while fixing on 1 precursor m/z and scanning the fragments. MS-MS plots demonstrated affinity of the heme for peptide or protein.

Hemin solutions were made in small amounts of 0.2 N ammonium hydroxide, with final dilution to 1 mg/mL and 70% acetonitrile, and added to 1 mmol/L BKPP. Oxygen was kept from solutions by N2 purging. Dithiothreitol was used at 2 to 5 times hemin on a molar basis to reduce the heme to hemin. Myoglobin was dissolved in 50 mmol/L ammonium bicarbonate (500 μmol/L). SDT at a 1.3-fold higher concentration than myoglobin was used to reduce the integral heme iron, followed by desalting on a Sephadex G-25 column.

Electrophysiology

Smooth muscle cells were isolated from resistance-size (~150 μm) rat cerebral arteries using an enzyme procedure that was similar to a procedure that has been previously described.21 Cos-1 cells were transfected with cloned rat cerebral artery smooth muscle cell slo (cbv1) or cbv1, in which CKACH was mutated to CKASR between amino acids 612 and 616. Channel currents were measured from inside-out membrane patches using an Axopatch 200B patch-clamp amplifier and a Digidata 1322A. Bath solution contained (in mmol/L): 140 KCl, 5.2 CaCl2, 1 MgCl2, 3 EGTA, 1.6 HEDTA, and 10 HEPES (pH 7.2), and pipette solution contained (in mmol/L): 140 KCl, 2 MgCl2, 2 CaCl2, and 10 HEPES (pH 7.4). The free [Ca2+]i bathing the intracellular membrane surface was 10 μmol/L, as confirmed with a Ca2+-sensitive and reference electrode (Corning). Following preparation, bath solutions were placed in a gas impermeant container and continually perfused through the patch-clamp chamber at a rate of 4 mL min−1. O2 pressure in the patch-clamp chamber was monitored using a PO2 electrode (Extech Instruments). Dilution of heme to a 100 nmol/L concentration in bath solution was accomplished by addition of 2 μmol/L dithionite. Dithionite (2 μmol/L) did not alter BKCa channel activity (data not shown). BKCa channel open probability (Po) was calculated from the following equation:  

\[
P_o = \frac{N_{Pl} \cdot P_{max}}{N_{Pl} + P_{max}}
\]

where N is the total number of channels in the patch (determined by application of 100 μmol/L free Ca2+ at +40 mV). Where appropriate, data were fit with a Hill equation:  

\[
P_o = P_{max} \left[ \text{Hemin} \right]^{n} \left[ \text{K}_d + \text{Hemin} \right]
\]

where \(P_{max}\) is the open probability, \(K_d\) is the dissociation constant, \(n\) is the Hill coefficient, and \(P_{max}\) is maximal \(P_o\).

cDNA Synthesis From Cerebral Artery Smooth Muscle Cells

Following isolation from rat basilar and middle-cerebral arteries,22 total RNA from 100 smooth muscle cells was isolated using an inverse microscope using Hoffmann optics and individually aspirated into Eppendorf tubes containing ~50 μL of PicoPure extraction buffer (Arcturus). Total RNA was treated with RNase-free DNase (RNase-Free DNase Reaction Buffer) and purified on an RNase-Free DNase Reaction Column (Arcturus). cDNA synthesis was performed on an Amplitron II-Thermocycler (Barnstead Thermolyne) using 2 U of Platinum TaqDNA polymerase (Invitrogen). Based on the amplified rat slo fragment sequences, we designed 2 primers: GSP1, cca tgc tgt ccc ac; GSP2, ctc att cct ccc ac; GSP3, gca agt gat gcc aaa gaa gtt a-3; reverse, 5’-atc tgg ctc tcc cag gag gtg-3’. PCR amplification was performed on an Ampliton II-Thermocycler (Barnstead Thermolyne) using 2 μL of the first strand cDNA in a final volume of 50 μL of system with 2 U of Platinum TaqDNA polymerase (Invitrogen). Amplification of rat cerebral artery smooth muscle cell slo1 (KC-NMA1) fragments was performed using the following polymerase chain reaction (PCR) primers: forward, 5’-gca gtt gcc gac gaa gag gtt a-3; reverse, 5’-act ctc tca ggc gat ccc c-3’. PCR amplification was performed on an Ampliton II-Thermocycler (Barnstead Thermolyne) using 2 μL of the first strand cDNA in a final volume of 50 μL of system with 2 U of Platinum TaqDNA polymerase (Invitrogen). Based on the amplified rat slo fragment sequences, we designed 2 primers: GSP1, cca tgc tgt ccc ac; GSP2, ctc att cct ccc ac; GSP3, gca agt gat gcc aaa gaa gtt a-3; reverse, 5’-atc tgg ctc tcc cag gag gtg-3’. PCR amplification was performed on an Ampliton II-Thermocycler (Barnstead Thermolyne) using 2 μL of the first strand cDNA in a final volume of 50 μL of system with 2 U of Platinum TaqDNA polymerase (Invitrogen). Based on the amplified rat slo fragment sequences, we designed 2 primers: GSP1, cca tgc tgt ccc ac; GSP2, ctc att cct ccc ac; GSP3, gca agt gat gcc aaa gaa gtt a-3; reverse, 5’-atc tgg ctc tcc cag gag gtg-3’. PCR amplification was performed on an Ampliton II-Thermocycler (Barnstead Thermolyne) using 2 μL of the first strand cDNA in a final volume of 50 μL of system with 2 U of Platinum TaqDNA polymerase (Invitrogen). Based on the amplified rat slo fragment sequences, we designed 2 primers: GSP1, cca tgc tgt ccc ac; GSP2, ctc att cct ccc ac; GSP3, gca agt gat gcc aaa gaa gtt a-3; reverse, 5’-atc tgg ctc tcc cag gag gtg-3’. PCR amplification was performed on an Ampliton II-Thermocycler (Barnstead Thermolyne) using 2 μL of the first strand cDNA in a final volume of 50 μL of system with 2 U of Platinum TaqDNA polymerase (Invitrogen). Based on the amplified rat slo fragment sequences, we designed 2 primers: GSP1, cca tgc tgt ccc ac; GSP2, ctc att cct ccc ac; GSP3, gca agt gat gcc aaa gaa gtt a-3; reverse, 5’-atc tgg ctc tcc cag gag gtg-3’. PCR amplification was performed on an Ampliton II-Thermocycler (Barnstead Thermolyne) using 2 μL of the first strand cDNA in a final volume of 50 μL of system with 2 U of Platinum TaqDNA polymerase (Invitrogen). Based on the amplified rat slo fragment sequences, we designed 2 primers: GSP1, cca tgc tgt ccc ac; GSP2, ctc att cct ccc ac; GSP3, gca agt gat gcc aaa gaa gtt a-3; reverse, 5’-atc tgg ctc tcc cag gag gtg-3’. PCR amplification was performed on an Ampliton II-Thermocycler (Barnstead Thermolyne) using 2 μL of the first strand cDNA in a final volume of 50 μL of system with 2 U of Platinum TaqDNA polymerase (Invitrogen). Based on the amplified rat slo fragment sequences, we designed 2 pr
tcc act cca tcc ggt cca to conduct 3’ RACE. Target 3’ cDNA ends were amplified by 2 rounds of PCR under optimal conditions using the Invitrogen 3’-RACE System.

**Full-Length slo1 Amplification and Sequence Analysis**

After comparing the sequences of slo1 3’ cDNA ends of rat cerebral artery smooth muscle cells, another 2 primers for first-round PCR were designed: 5 out, tcg tcc tct tct gcc tcc tgg gcc g; 3 out, acg tca cca tta agt cag tgg gtc ag. For the second round of PCR, primers were as follows: 5 in, gcc gcc gcc gcc gcc gcc gcc gcc; 3 in, cca gtc tca tca tga act tc. Target cDNAs were amplified by nested PCR in optimal conditions; first-round PCR was conducted in a total volume of 50 μL containing the following: 20 mmol/L Tris-HCl (pH 8.4); 50 mmol/L KCl; 2 mmol/L MgCl2; 300 nmol/L primer 5 out; 200 nmol/L of each dNTP; 2 μL cDNA; and 1.5 U of DNA polymerase (Expand High Fidelity PCR system; Roche).

A band of ~3.6 kb was amplified, rescued, and ligated to the pCR-XL-Topo vector (Invitrogen). Using restriction enzyme mapping and sequence analysis, we screened 2 slo1 isoforms (from ~18 clones) that contained the 3.6-kb insert. After full sequencing, information corresponding to the 2 isoforms of rat cerebral artery myocyte slo1 (termed cbv1 and cbv2) was deposited into GenBank (AY330293 and AY330294).

**Cbv1 Insertion for Mammalian Expression and Mutagenesis**

PCR primers were designed starting from the second putative Met: forward, cac caa gat gga tgc gct cat cat ccc; reverse, tct gta aac cat tcc ttt ttc tgt gtc ggc. A Kozak sequence was introduced at the 5’ end to improve expression in mammalian cells. Amplified PCR fragments were then ligated directly to the pcDNA3/V5/His-TOPO vector (Invitrogen).

A double mutation was introduced in cbv1 to eliminate the heme binding motif (CKACH to CKASR), using Quickchange (Stratagene). The oligo sequences used were as follows: forward, gca ttt ttt ctg ttt gtc gcg. A Kozak sequence was introduced at the 5’ end to improve expression in mammalian cells. Amplified PCR fragments were then ligated directly to the pcDNA3/V5/His-TOPO vector (Invitrogen).

**Cell Culture and Transfection**

Cos1 cells were maintained in DMEM (Cellgro) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin. pCDNA3 encoding cbv1 and pEGFP-C3 were cotransfected into Cos1 cells using FuGENE 6 (Roche). Cells were cultured at 37°C in 95% air/5% CO2/1% H2. Cells were transfected as follows: forward, gcc ttt ttt tac tgc ggc tct cgt gat gac gcg; reverse, gtc agc tca tca cga gag gcc tgt tgc taa aat gc. Fidelity of desired mutations and absence of unwanted mutations was verified by sequencing.

**Statistics**

Summary data are expressed as mean±SEM. ANOVA, paired or unpaired Students t, Student–Newman–Keuls, and/or Wilcoxon matched-pairs signed-rank test were performed according to experimental design and data distribution (Kolmogorov and Smirnov distance). P<0.05 was considered significant.

An expanded Materials and Methods section is provided in the online data supplement available at http://circres.ahajournals.org.

**Results**

To investigate whether CO can interfere with binding of heme to the BKCa channel heme-binding domain, a novel thin-layer chromatography (TLC) assay was used. A 23 amino acid peptide corresponding to the hso sequence that contained the conserved heme-binding domain (CKACH) was constructed (BKCa channel pocket peptide, BKPP). BKPP was spotted on silica gel TLC plates and solvent progression carried the peptide to immobile heme where it either bound or migrated through. Sample TLC chromatograms illustrating passage of BKPP in a N2 atmosphere or a CO containing atmosphere are shown in Figure 1A. Relative BKPP migration through reduced (ferrous, Fe2+) heme (hereafter termed heme) or oxidized (ferric, Fe3+) heme (termed hemin) were compared with freely migrating peptide (no heme or hemin) on the same plate (Figure 1B and 1C). In a N2 atmosphere, only ~12% of BKPP passed through heme, whereas in a CO-containing atmosphere, BKPP passage increased to ~63%. In contrast, CO did not alter the block of BKPP migration by hemin, to which CO cannot bind. To investigate whether heme blocked BKPP migration by binding to the heme-binding domain, a peptide containing a double mutation in the heme-binding domain (CKACH to CKASR, termed BKMP) was constructed. A similar mutation has been shown to abolish heme inhibition of BKCa channels. Even in...
a purely N₂ atmosphere, heme did not prevent migration of BKMP.

Spectrophotometry was used as a complimentary approach to investigate heme to peptide molecular interactions. Heme produced an absorbance peak at ~390 nm (the Soret band). Addition of CO (20 to 100 μmol/L) to heme produced a concentration-dependent increase in a peak at ~410 nm, indicating CO binding to heme (Figure 2A through 2D). In contrast, addition of CO (20 to 100 μmol/L) to hemin did not alter the spectra, suggesting no interaction (data shown for 20 μmol/L CO in Figure 2A). Addition of BKPP (20 μmol/L) to a heme–CO mixture, or addition of CO to a heme–BKPP mixture, reduced the 410-nm absorption peak (Figure 2C and 2D). In contrast, BKPP (20 to 100 μmol/L) alone did not alter heme spectra (data not shown).

To specifically identify heme bound to BKPP, electrospray ionization mass spectrometry was used. Figure 3A unequivocally identifies a molecule comprised of heme bound to BKPP, the theoretical mass matching the experimental. To determine relative binding of heme to BKPP and to BKMP, we measured the proportion of peptide with adherent heme. Heme was added to peptide in a 2-to-1 ratio. As expected, many more BKPP molecules bound heme (28±6%) than did BKMP molecules (7±2%), consistent with the TLC results.

Next, MS-MS techniques were used to investigate the effect of CO on the binding of heme within myoglobin, a native heme protein, and the effect of CO on the binding of heme to BKPP. For myoglobin, collision energy curves show that CO causes release of heme from the protein at lower energies than when CO is absent (Figure 3B). Heme and BKPP produce a multicomponent curve (Figure 3C). Some heme binds loosely with only low collision energies required to separate it from BKPP. The strength of this low-affinity binding is increased by CO. However, some heme binding is much stronger, and this high affinity binding is abolished by CO (Figure 3C, inset).

Because CO binds to heme, and heme binds to BKCa channels, CO may activate BKCa channels by binding to channel associated heme. Therefore, the activity of single cerebral artery smooth muscle cell BKCa channels were recorded using the inside-out patch-clamp configuration with 10 μmol/L free Ca²⁺ at the intracellular membrane surface. At +40 mV, hemin reduced BKCa channel open probability (Pₒ) with a half-maximal inhibitory concentration of 5.1 nmol/L and a Hill coefficient of 1.03 (Figure 4A and 4B). BKCa channel inhibition by hemin (100 nmol/L) was similar at −40 (to 3.9±1.2% of control; n=7, P<0.05) and +40 mV (Figure 4A through 4C).

Subsequent electrophysiological experiments were performed with a physiological intracellular Po₂ of 20 mm Hg that would lessen oxidation of reduced heme to hemin.
Reducing O₂ from 150 mm Hg (ambient air) to 20 mm Hg decreased mean BK<sub>Ca</sub> channel P<sub)o</sub> from 0.32±0.08 to 0.12±0.04, or to 45.7±6.8% of control (n=17, P<0.05), consistent with previous reports. With a PO<sub>2</sub> of 20 mm Hg, hemin and heme (100 nmol/L each) reduced mean BK<sub>Ca</sub>-channel P<sub)o</sub> similarly (Figure 5A and 5C). More importantly, and consistent with the spectra and TLC data, CO activated BK<sub>Ca</sub> channels that were inhibited by heme, but CO did not alter the activity of channels inhibited by hemin (Figure 5A and 5C). Moreover, following CO-induced BK<sub>Ca</sub> channel activation, addition of heme further activated BK<sub>Ca</sub> channels, suggesting CO–heme is an activator (Figure 5B and 5D). In contrast, heme blocked BK<sub>Ca</sub> channels when applied in the continued presence of CO (Figure 5B and 5D).

To investigate whether CO activates BK<sub>Ca</sub> channels by binding to a subunit-associated, endogenous heme, BK<sub>Ca</sub> channels were cloned from smooth muscle cells of rat cerebral arteries and termed cbv1 and cbv2. Sequence analysis of full-length cbv1 (GenBank accession no. AAP82453) and cbv2 (GenBank accession no. AAP82454) indicated that the conserved heme-binding domain (CKACH) is located between amino acids 612 and 616. Cbv1 and cvb2 channels expressed in cos-1 cells exhibited properties similar to native cerebral artery smooth muscle cell BK<sub>Ca</sub> channels, including slope conductance, voltage-dependence, Ca<sup>2+</sup> sensitivity, and pharmacology (online data supplement). As illustrated in Figure 6, wild-type cbv1 channels were inhibited by heme (500 nmol/L and 50 μmol/L) and activated by CO, consistent with regulation of native arterial smooth muscle cell BK<sub>Ca</sub> channels (Figure 5). In addition, CO applied in the continued presence of heme was a more effective cbv1 channel activator than CO alone. In contrast, heme-binding domain mutated cbv1 channels were insensitive to heme (500 nmol/L and 50 μmol/L), CO (10%), and the combination of CO+heme (Figure 6B and 6D).

**Discussion**

The current report provides direct experimental evidence for a mechanism by which CO interacts with and activates BK<sub>Ca</sub> channels. Data indicate CO activates BK<sub>Ca</sub> channels by binding to endogenous channel-bound heme and altering the interaction between heme and the heme-binding domain.

CO activates arterial smooth muscle and carotid body BK<sub>Ca</sub> channels in excised membrane patches and activates cso and
mslo channels when expressed in the absence of auxiliary β subunits.11,18,19,24 Thus, β subunits are not required for CO-induced BKCa channel activation, indicating that CO acts on the pore-forming α subunit itself or another tightly associated regulatory molecule. The hypothesis that CO may influence activity by binding directly to BKCa channels has been proposed.25,26 However, under physiological conditions CO should not bind directly to proteins, and no direct experimental evidence or chemical mechanism to support such binding and activation has been obtained.12

Cellular heme is produced in the reduced state and, being hydrophobic, incorporates readily into lipid bilayers.27 Data indicate that native ferrous heme bound to amino acids 612 to 616 of the BKCa channel provides a receptor for CO. Within the heme-binding domain is an integral histidine that binds the iron center of heme. Mutation of the histidine within the heme-binding domain prevented hemin-induced BKCa channel inhibition.9 In other studies, chemical modification of histidines abolished CO-induced BKCa channel activation.26,28 Thus, to investigate the importance of the heme-binding domain in CO-induced BKCa channel activation, cbv1 channels were constructed that contained a 2 amino acid mutation in the heme-binding domain, similarly to the mutation present in the BKMP (ie, from CKACH to CKASR). Mutation of the histidine and the proximal cysteine that provides disulphide bridge binding with the heme porphyrin markedly attenuated heme–BKPP binding, the ability of heme to block BKCa channels, and the ability of CO to activate BKCa channels. In addition, BKCa channels with native heme-binding domains are insensitive to CO if the heme-binding domain is occupied with hemin that does not bind CO. Binding of CO to heme reduces the inhibitory interaction and may generate an activator configuration, leading to the previously reported increase in BKCa channel Ca2⁺ sensitivity.19 In smooth muscle cells, an increase in BKCa channel Ca2⁺ sensitivity would enhance coupling to Ca2⁺ sparks and increase transient BKCa current frequency and amplitude, leading to membrane hyperpolarization and, ultimately, vasodilation.17,19 CO-to-heme binding is a readily reversible carbon monooxygenation that is dependent on CO partial pressure. Therefore, activation of cellular CO production would increase Pco2, leading to an increase in CO to heme binding and BKCa channel activation, whereas reducing Pco2 would lead to the removal of CO from channel-bound heme and a decrease in BKCa channel activity. CO changed the binding curve of heme to BKPP; therefore, its shape is similar to that of myoglobin with CO. As a short sequence, BKPP does not have the binding strength of native myoglobin; therefore, the curve is shifted left. Although the shape of the N2 (non-CO) curves are different for myoglobin and BKPP, they both have higher affinity binding than their CO counterparts. The multicomponent curve of BKPP and heme strongly suggests different kinds of heme to BKPP binding can occur under the nonphysiological conditions necessitated by MS. Some heme binds relatively loosely, perhaps nonspecifically, and low collision energies separate it from the peptide. This low affinity binding is actually strengthened by CO. Beyond this, 10% of the heme adheres much more strongly to the peptide, and this adherence is virtually eliminated by CO.

Hemin has been demonstrated to regulate heterologously expressed hso1 channels,9,20 but whether hemin or heme regulate native BKCa channels was unknown. Thus, we investigated hemin, heme, and CO regulation of BKCa channels in smooth muscle cells of small cerebral arteries that regulate blood pressure and flow. In smooth muscle cells, BKCa channels are activated by Ca2⁺ sparks that elevate intracellular Ca2⁺ within the micromolar concentration...
Therefore, regulation was studied with 10 μmol/L Ca^{2+} present at the BK_{Ca} channel inner-membrane surface. Data show that the half-maximal inhibitory concentration of heme for arterial smooth muscle BK_{Ca} channels was approximately 1-order of magnitude lower than that previously described for hslo channels, suggesting higher heme sensitivity. One explanation for different heme sensitivity may be a valine for isoleucine substitution 3 amino acids distal to the histidine in the heme-binding domain of the rat BK_{Ca} channel (GenBank accession nos. AAA92290 and AY330293). Although just outside the conserved heme-binding domain, this substitution may improve heme accessibility to critical amino acids. However, native rat cerebral artery smooth muscle BK_{Ca} channels were also noticeably more sensitive to hemin and heme than cbv1 channels when expressed in cos cells. Another potential explanation for the

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**Figure 5.** CO reverses BK_{Ca} channel inhibition by heme but not by hemin. A, Original recordings of arterial smooth muscle BK_{Ca} channels at -40 mV. Heme blocked BK_{Ca} channels, and subsequent addition of CO (10%) induced channel activation. Hemin also blocked BK_{Ca} channels, but subsequent addition of CO did not alter channel activity. B, Original recordings illustrating BK_{Ca} channel activation by CO and subsequent effects of heme or hemin addition. C, Summary data indicating CO reverses inhibition of BK_{Ca} channels by heme (n = 6) but does not alter the activity of channels inhibited by hemin (n = 4). *P<0.05 compared with heme. D, Mean effects of CO (10%, n = 11) and subsequent heme (n = 6) or hemin (n = 5) addition. *P<0.05 compared with 100%, †P<0.05 compared with CO.

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**Figure 6.** Mutation of the BK_{Ca} channel heme-binding domain abolishes heme inhibition and CO activation. A, Original recordings of cbv1 BK_{Ca} channels measured in inside-out patches illustrating inhibition by heme (500 nmol/L) and activation by CO (10%). WT indicates wild type. B, A 2 amino acid mutation of the heme-binding domain (CH to SR [CH-SR]) abolishes heme-induced cbv1 channel inhibition and CO activation. C, Summary data illustrating effects of heme (500 nmol/L, n = 6; 5 μmol/L, n = 6), CO (n = 4), and CO+heme (n = 4) on cbv1 channels. D, Mean data illustrating effects of heme (500 nmol/L, n = 8; 5 μmol/L, n = 8), CO (n = 8), and CO+heme (n = 13) on mutant cbv1 channels. *P<0.05 compared with 100%, †P<0.05 compared with CO.
difference in heme sensitivity is that cerebral artery smooth muscle cells are enriched in β1 subunits. Although β subunits are not obligatory for heme inhibition, the presence of these accessory channel subunits may increase heme sensitivity. The fact that both heme and β1 subunits alter BKCa channel Ca2+ sensitivity1,2,20,31 is consistent with this hypothesis.

HO and BKCa channels are membrane colocalized.11 Conceivably, HO activation may not only generate CO, a BKCa channel activator, but may also reduce membrane associated heme, a BKCa channel blocker, to generate the CO. Both of these effects would elevate BKCa channel activity. Thus, it is possible that compartmentalization of the CO generator (HO), CO receptor (heme), and downstream target (BKCa channel) may regulate cellular excitability through more than one signaling pathway.

O2 regulates BKCa channel activity by acting as a reactant for HO catabolism of heme.11 However, O2 also regulates BKCa channel activity when NADPH, another obligatory reactant, is absent, and thus HO cannot metabolize heme.11 Thus, additional HO-independent O2 sensors appear to exist for BKCa channels. Conceivably, heme may also act as a binding site for other gaseous messengers, including O2. As such, heme may act as a receptor that regulates BKCa channel activity and thus cellular excitability in response to diverse physiological and pathological stimuli. In summary, data here indicate that heme is a functional CO receptor for BKCa channels.

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Results

Cloning and functional characterization of BK$_{Ca}$ channel-forming (rslo cbv) subunits from rat cerebral artery smooth muscle cells

Smooth muscle cells were isolated from adult rat basilar and middle cerebral arteries as described elsewhere $^1$. Following isolation, smooth muscle cells were individually collected using an enlarged patch-clamp micropipette; total RNA was obtained from ~100 cells. Following reverse transcription, 3’-RACE and PCR were conducted and amplicons were sequenced at the University of Tennessee Molecular Resource Center. Using restriction enzyme mapping and sequencing, two isoforms were screened (from ~18 clones) that contained the 3.6 kb insert attributable to rslo. After full sequencing, information corresponding to these rslo (termed cbv1 and cbv2) were deposited into Genbank (AY330293 and AY330294). After insertion into the pOX $Xenopus$ expression vector, cbv1 and cbv2 cDNAs were linearized with NotI, and transcribed using T3 pol. cRNAs were injected as described for other slo $^2$. After expression in $Xenopus$ oocytes, rslo currents were acquired and analyzed as previously described for other slo channels in the same expression system $^2, ^3$. Cbv1 and cbv2 rslo currents were obtained from inside-out patches more than 5 minutes after patch excision from the cell. Pipette solution contained (in mM): Kgluconate 130, CaCl$_2$ 5.2, MgCl$_2$ 2.3, EGTA 5, HEDTA 1.6, HEPES 10 (free [Ca$^{2+}$],~11 µM). Bath solution contained (in mM): Kgluconate 130, CaCl$_2$ 3.84, MgCl$_2$ 1, EGTA 5, HEPES 10 (free [Ca$^{2+}$],~0.3 µM). Cbv1 and cbv2 rslo currents obtained from inside-out patches showed: 1) ohmic behavior between -60 and 40 mV, with slope conductances of 220±25 (cbv1) and 201±17 (cbv2) pS in symmetric 130 mM K$^+$, respectively. For both rslo channels, unitary currents displayed a Nernst shift of ~38 mV in $V_{i=0}$ when [K$^+$], was changed
from 130 to 30 mM (Na⁺ substituting for K⁺). Together, these data indicate that cbv1 and cbv2 channels display high permeability and selectivity for K⁺ over Na⁺; 2) an elevation in channel steady-state activity (NP₀, calculated from all points amplitude histograms, as described in ⁴) with more positive transmembrane potentials: 11±1 and 11±2 mV/e-fold change in NP₀ for cbv1 and cbv2, respectively. Data are given as mean±SEM (n=3-4); 3) an increase in NP₀ (× >10 times) with an elevation in [Ca²⁺]ᵢ from subµM to µM (Supplemental Figure 1). Data from outside-out patches demonstrated that: 1) external TEA (1 mM) and iberiotoxin (100 nM) caused a flickery and a slow current block, respectively, almost totally abolishing rslo current (not shown), as described for native BKₖCa channels ⁵; 2) external 17β estradiol (5-10 µM) increased currents (× ~5 times) only when rslo channels were coexpressed with β₁ subunits (Supplemental Figure 2), as previously reported for bslo channels ².

In summary, full-length slo subunits were cloned from freshly isolated rat cerebral artery smooth muscle cells. Rslo expression led to single channel events that displayed all major characteristics of BKₖCa unitary currents. These data have been sent for communication at the Society for Neuroscience 2005 annual meeting (Liu, J, Liu, P, Crowley, JJ, Asuncion-Chin, MT, and Dopico, AM. “Cloning and functional characterization of BKₖCa channel-forming (rslo cbv) subunits from rat cerebral artery myocytes”).
Detailed Materials and Methods

Thin layer chromatography

Native heme-binding pocket peptide (DAEVKRAFFYCKACHDDITDPK, BKPP) and double mutant (C to S and H to R) peptide (DAEVKRAFFYCKASRDDITDPK, BKMP) were dissolved in water (stock solution: 1 mM). Peptides (10 µl) were applied 2 cm from the bottom edge of 5 x 20 cm silica gel 60 plates and allowed to dry. Deoxygenated Krebs (dKrebs) was prepared by vigorously diffusing N₂ through Krebs solution in a parafilm-sealed Erlenmeyer flask. Fe²⁺-heme (500 µM) solution was prepared by dissolving Fe³⁺-hemin in basic (pH 8) dKrebs and reducing it with sodium dithionite (SDT, added to a final concentration of 2 mM). Exposure of porphyrins to light and air was minimized. Heme (10 µl, 1 mM) was applied 3 cm from the bottom edge (1 cm above the peptide) and the plate was immediately placed in a gas controlled, environmental chamber development tank. The chromatography solvent was ethanol/isopropanol/PBS (15/15/70). Solvent migration time was 2 hr in atmospheres of either 100% N₂, 100% CO, or 10% CO and 90% N₂ (data for 100% and 10% CO were the same and thus, combined as CO). Under these conditions heme does not move but the peptide migrates into the heme spot. If the peptide binds to the heme, its migration will be either stopped or slowed. Plates were dried in room air and peptides stained with naphthol blue black (500 mg dissolved in methanol: acetic acid: water (45: 10:45), 3 min), rinsed with water (3 min), and destained with methanol:acetic acid:water (90:2:8)). Line scans were obtained and intensity quantified using a digital camera, digital imaging and densitometry with NIH Image software.
**Spectrophotometry**

Carbon monoxide was prepared as a saturated solution (1 mM) by vigorously bubbling distilled water with 100 % CO for 2 hours under a headspace gas of 100 % CO. CO concentrations in the solutions were measured by GC-MS. Under reducing conditions (0.5mM SDT and anoxic Krebs), CO (as saturated solution in water, 1mM) was added to heme to make final concentrations of 20, 40, and 100 µM. The stock solution of BKPP peptide (1 mM), corresponding to the heme-binding pocket of the BK channel, was prepared in water and stored at –20°C. For experimentation, BKPP peptide was dissolved in dKrebs to final concentrations of 20-100 µM. All solutions were protected from air and light, and the spectra were taken immediately after mixing the solutions in a parafilm-sealed optical cuvette with the head space purged with N₂. To investigate interactions between heme and CO under reducing conditions, the absorption spectrum of heme solution was recorded first, then CO was added to make a final concentration of 20-100 µM in the cuvette, and spectra were recorded again. To investigate the effects of BKPP on heme - CO interactions under reducing conditions, BKPP was dissolved in dKrebs to make final concentrations of 20-40 µM and was added to the heme solution a) in the absence of CO, b) immediately before CO, and c) immediately after CO, and the absorption spectra of heme solutions were recorded. To investigate interactions among hemin, CO, and BKPP under non-reducing conditions, the above experimental protocol was repeated in the absence of SDT. Scans were performed using an Ultraspec 2100 UV/visible spectrophotometer (Amersham, Piscataway, NJ) and analyzed using Biochrom Data Capture spreadsheet interface software. Each experiment was repeated at least 5 times.
**Mass Spectrometry**

A Micromass Qtof2 mass spectrometer with a nano-electrospray ionization probe and MassLynx software (version 3.5) was used for mass spectrometry (MS). For MS, collision energies of 5 or 8V were used and the instrument scanned at 100 to 2000 m/z for all solution ions. The voltages were kept very low to enable peak visualization of weakly bound ion complexes. Spray needle voltage was 1.8 kV, cone voltage was 5V. Solutions were sprayed in 25 mM ammonium bicarbonate, 12 to 25 % acetonitrile. Syringe infusion rate through a 30 µm orifice spray needle was 0.4 µl/min. In all cases, stable protein or peptide charge states were formed, and the protein was not denatured. For MSMS, progressively increasing voltages were used while fixing on one precursor m/z and scanning the fragments. MSMS plots demonstrated affinity of the heme for peptide or protein.

Hemin solutions were made in small amounts of 0.2N ammonium hydroxide, with final dilution to1mg/ml and 70% acetonitrile. Oxygen was kept from solutions by N$_2$ purging. Dithiothreitol (DTT) was used at 2 to 5 times hemin on a molar basis to reduce the hemin to heme. Myoglobin was made to 500µM in 50mM ammonium bicarbonate. SDT at a 1.3-fold higher concentration than myoglobin was used to reduce the integral heme iron, followed by desalting on a Sephadex G-25 column. Amounts of protein/peptide electrosprayed varied from 20 to 150 µM.

**Electrophysiology**

Smooth muscle cells were isolated from resistance-size (~150 µm) rat cerebral arteries as previously described. Cells were used within 8 hours of isolation. Cos-1 cells were transfected with cloned rat cerebral artery smooth muscle channels (cbv1) or cbv1 in which CKACH was
mutated to CKASR between amino acids 612 and 616, as in the BKPP and BKMP peptides.

Channel currents were measured from inside-out membrane patches using an Axopatch 200B patch-clamp amplifier and a Digidata 1322A. Bath solution contained (in mM): 140 KCl, 5.2 CaCl$_2$, 1 MgCl$_2$, 5 EGTA, 1.6 HEDTA, and 10 HEPES (pH 7.2), and pipette solution contained (in mM): 140 KCl, 2 MgCl$_2$, 2 CaCl$_2$, and 10 HEPES (pH 7.4). The free [Ca$^{2+}$] bathing the intracellular membrane surface was 10 µM, as confirmed with a Ca$^{2+}$-sensitive and reference electrode (Corning). CO was prepared as a saturated, deoxygenated solution (1 mM) and diluted as required. Low O$_2$ solutions were prepared from anoxic bath solution that was made by purging with 100% N$_2$. Following preparation, bath solutions were placed in a gas impermeant container and continually perfused through the patch-clamp chamber at a rate of 4 ml min$^{-1}$. O$_2$ pressure in the patch-clamp chamber was monitored using a PO$_2$ electrode (Extech Instruments) and confirmed that gas exchange with room air was minimal at this perfusion rate. Dilution of heme to a 100 nM concentration in bath solution was accompanied by addition of 2 µM dithionite. Dithionite (2 µM) did not alter BK$_{Ca}$ channel activity (data not shown). K$_{Ca}$ channel open probability (P$_o$) was calculated from the following equation: P$_o$ = NP$_o$/N, where N is the total number of channels in the patch (determined by application of 100 µM free Ca$^{2+}$ at +40 mV). Where appropriate, data were fit with a Hill equation: P$_o$=P$_{max}$ [Hemin]$^n$/(K$_d$)$^n$ + Hemin]$^n$, where P$_o$ is the open probability, K$_d$ is the dissociation constant, n is the Hill coefficient, and P$_{max}$ is maximal P$_o$.

cDNA synthesis from cerebral artery smooth muscle cells

Individual smooth muscle cells were isolated from rat basilar and middle-cerebral arteries using enzymes, as previously described$^1$. Smooth muscle cells were identified under an inverted...
microscope using Hoffmann optics and individually aspirated into glass micropipettes (≤10 µm diameter). Smooth muscle cells were released into Eppendorf tubes containing ~50 µl of PicoPure extraction buffer (Arcturus), and stored at -80°C. Total RNA from ~100 smooth muscle cells was isolated using the PicoPure RNA Isolation Kit (Arcturus).

First strand cDNA was synthesized from the total RNA using an oligo(dT)-primed 20 µl reaction by SuperscriptII Reverse transcriptase (Invitrogen) under standard conditions (final concentrations: 8 mM Magnesium acetate, 50 mM Tris-HCl, 75 mM KCl, 500 µM each dNTP, 25µM oligo(dT) primer and 7.5 U SuperscriptII Reverse transcriptase).

**PCR amplification of slo1 conserved fragment and 3’-RACE**

Amplification of rat cerebral artery smooth muscle cell slo1 (KCNMA1) fragments was performed using the following PCR primer pairs: forward, 5’-gca agt gat gcc aaa gaa gtt a-3’ and reverse, 5’-atc tgt cca ttc cag gag gt-3’. PCR amplification was performed on an AMPLITRON II-Thermocycler (Barnstead Thermolyne) using 2 µl of the first strand cDNA in a final volume of 50 µl containing 2 U Platinum TaqDNA polymerase (Invitrogen), 200 µM of each dNTP, 1.5 mM MgCl₂, 40 mM KCl, 16 mM Tris-HCl, and a primer concentration of 200 mM each. The cycling protocol for the PCR reaction was: 94 ºC for 2 min, [94 ºC for 30 s, 52 ºC for 45 s and 72 ºC for 50 s] x 25 cycles, and 72ºC for 2 min. Amplicons were automatically sequenced at the University of Tennessee Health Science Center Molecular Resource Center. Based on these rat KCNMA1 fragment sequences, we designed two primers: GSP1, cca gtc tgt ctc att cct ccc ac, and GSP2, ctg tcc act cca tcc cgt cca to conduct 3’RACE. Target 3’ cDNA ends were amplified by two rounds of PCR under optimal conditions using the 3’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen).
Full-length slo1 amplification and sequence analysis

After comparing the sequence of slo1 3’ cDNA ends of rat cerebral artery smooth muscle cells, another two primers for first round PCR were designed: 5 out, tcc tcc tct tcc tcc tcg tcc tcg g, and 3 out, acg tca cca ttt atg cag ttt gtc ag. For the second round of PCR, primers were: 5 in, gtc cac gag ccc aag atg gat gcc c, and 3 in, cct ggg aat caa cat tca tct tca act tc. Target cDNAs were amplified by nested PCR in optimal conditions according to the following protocol: first round PCR was conducted in a 0.5-ml microcentrifuge tube containing: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl2, 300 nM primer 5out, 300 nM primer 3out, 200 µM of each dNTP, 2 µl cDNA, and 1.5 units of DNA polymerase (Expand High Fidelity PCR system, Roche) for a total volume of 50 µl. The cycling protocol for PCR was: 94 °C for 1 min; denaturation, 94°C for 30 s; annealing of primers, 61 °C for 30 s with a decrease of 0.3 °C/cycle; primer extension, 72°C for 1 min x 10 cycles; denaturation, 94°C for 30 s; annealing of primers, 58 °C for 30 s; extension, 8 s/cycle; primer extension, 72 °C for 1 min x 15 cycles, followed by final extension, 72 °C for 5 min. Second round PCR was conducted following a similar protocol.

A band of ~3.6 kb was amplified, rescued and ligated to the pCR-XL-Topo vector (Invitrogen). Using restriction enzyme mapping and sequencing analysis, we screened two slo1 isoforms (from ~18 clones) that contained the 3.6 kb insert. After full sequencing, information corresponding to the two isoforms of rat cerebral artery smooth muscle cell slo1 (termed cbv1 and cbv2) was deposited into Genbank (AY330293 and AY330294).
Cbv1 insertion for mammalian expression and mutagenesis

PCR primers were designed starting from the second Met: forward, cac caa gat gga tgc gct cat cat ccc, and reverse, tct gta aac cat ttc tt ttt ctg ttt gtc gcg. A Kozak sequence was introduced at the 5’ end to improve expression in a mammalian expression system. Amplified PCR fragments were then directly ligated to the pcDNA3/V5/His-TOPO vector (Invitrogen).

Mutagenesis of cbv1 was conducted using the Quickchange system (Stratagene) in order to eliminate the heme binding motif (CKACH) by introducing a double mutation (CKASR). Oligo sequences used for mutagenesis were: forward, gca ttt ttt tac tgc aag gcc tct cgt gat gac gtc ac, and reverse, gtg acg tca tca cga gag gcc ttg cag taa aaa aat gc. The fidelity of the desired mutations and the absence of unwanted mutations were verified by sequencing.

Cell culture and transfection

Cos1 cells were maintained in DMEM (Cellgro) supplemented with 10 % FBS (Gibco) and 1 % penicillin/streptomycin. pCDNA3 encoding cbv1 and pEGFP-C3 were co-transfected into cos1 cells using FuGENE 6 (Roche). Cells were cultured at 37 °C in a 95% air / 5 % CO2 atmosphere and used between 24 and 96 hours following transfection. eGFP was excited at 450-490 nm and emitted light between 500 and 550 nm was observed. Transfected cells were positively identified due to their green fluorescence.

Statistics

Summary data are expressed as mean ± standard error of the mean. ANOVA, paired or unpaired Students t , Student-Newman-Keuls, and/or Wilcoxon matched-pairs signed-ranks test were
performed according to experimental design and data distribution (Kolmogorov and Smirnov distance).
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Online Figure 2. Bath application of 17β-estradiol (10 µM) fails to regulate homomeric rslo cbv1 channels (A), but reversibly increases β₁+rslo cbv1 channel steady-state activity (NP₀) (B). Similar results were obtained when 17β-estradiol action was evaluated on β₁+rslo cbv2 vs. homomeric rslo cbv2 channels. Channel NP₀ was continuously recorded during voltage-clamp conditions (V=-20 mV) and [Ca²⁺]i ≈ 100 nM. Channel openings are shown as downward deflections, and arrows indicate the zero current level. The estradiol concentration is maximal for β₁+hslo channel activation 7. Pipette solution contained (mM): 130 Kgluconate, 4.6 CaCl₂, 2.5 MgCl₂, 5 EGTA, 1.6 HEDTA, 10 HEPES. The bath solution contained (mM): 130 Kgluconate, 5.2 CaCl₂, 2.3 MgCl₂, 5 EGTA, 1.6 HEDTA, and 10 HEPES.