Carbon monoxide activates K\textsubscript{Ca} channels in newborn arteriole smooth muscle cells by increasing apparent Ca\textsuperscript{2+} sensitivity of \(\alpha\)-subunits

Qi Xi, Dilyara Tcheranova, Helena Parfenova, Burton Horowitz, Charles W. Leffler, and Jonathan H. Jaggar. Carbon monoxide activates K\textsubscript{Ca} channels in newborn arteriole smooth muscle cells by increasing apparent Ca\textsuperscript{2+} sensitivity of \(\alpha\)-subunits. Am J Physiol Heart Circ Physiol 286: H610–H618, 2004. First published October 16, 2003; 10.1152/ajpheart.00782.2003.—Carbon monoxide (CO) is a gaseous vasodilator produced by many cell types, including endothelial and smooth muscle cells. The goal of the present study was to investigate signaling mechanisms responsible for CO activation of large-conductance Ca\textsuperscript{2+}-activated K\textsubscript{+} (K\textsubscript{Ca}) channels in newborn porcine cerebral arteriole smooth muscle cells. In intact cells at 0 mV, CO (3 \(\mu\)M) or CO released from dimanganese decarboxyl) (10 \(\mu\)M), a novel light-activated CO donor, increased K\textsubscript{Ca} channel activity 4.9- or 3.5-fold, respectively. K\textsubscript{Ca} channel activation by CO was not blocked by 1-H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (25 \(\mu\)M), a soluble guanylyl cyclase inhibitor. In inside-out patches at 0 mV, CO shifted the Ca\textsuperscript{2+} concentration-response curve for K\textsubscript{Ca} channels leftward and decreased the apparent dissociation constant for Ca\textsuperscript{2+} from 31 to 24 \(\mu\)M. Western blotting data suggested that the low Ca\textsuperscript{2+} sensitivity of newborn K\textsubscript{Ca} channels may be due to a reduced \(\beta\)-subunit-to-\(\alpha\)-subunit ratio. CO activation of K\textsubscript{Ca} channels was Ca\textsuperscript{2+} dependent. CO increased open probability 3.7-fold with 10 \(\mu\)M free Ca\textsuperscript{2+} at the cytosolic membrane surface but only 1.1-fold with 300 nM Ca\textsuperscript{2+}. CO left shifted the current-voltage relationship of csl\(\alpha\)-currents expressed in HEK-293 cells, increasing currents 2.2-fold at +50 mV. In summary, data suggest that in newborn arteriole smooth muscle cells, CO activates low-affinity K\textsubscript{Ca} channels via a direct effect on the \(\alpha\)-subunit that increases apparent Ca\textsuperscript{2+} sensitivity. The optimal tuning by CO of the micromolar Ca\textsuperscript{2+} sensitivity of K\textsubscript{Ca} channels will lead to preferential activation by signaling modalities, such as Ca\textsuperscript{2+} sparks, which elevate the subsarcolemmal Ca\textsuperscript{2+} concentration within this range.

patch-clamp electrophysiology; cerebrovascular circulation

Disorders of the cerebrovascular circulation in the newborn period are major causes of morbidity and mortality and can result in life-long disabilities in survivors. In contrast to our understanding of signaling mechanisms that control the adult circulation, knowledge of mechanisms that regulate the perinatal cerebrovascular circulation is limited. Recent studies have indicated that physiological regulation of the newborn vasculature is very different when compared with that of the adult. When considering the physiological functions of vascular smooth muscle, several age-related differences have been described, including those relating to resting membrane potential (8), voltage-dependent Ca\textsuperscript{2+} channels (2), intracellular Ca\textsuperscript{2+} dynamics (29), localized intracellular Ca\textsuperscript{2+} release events (14), and sensitivity to cGMP (33). Thus signal transduction mechanisms characterized in the adult circulation may not extrapolate to the newborn. These differences require independent studies to be performed to characterize the physiology of the newborn circulation.

Recent evidence suggests that carbon monoxide (CO) is an important paracrine and autocrine gaseous relaxing factor in the newborn and adult cerebral and systemic circulations (30, 44). CO is generated endogenously by the metabolism of heme by heme oxygenase (HO) (30). HO-1 and HO-2 (48), which are products of different genes, are found in many cell types, including arterial smooth muscle and endothelial cells (7, 28, 48). Arterial smooth muscle cells and endothelial cells produce HO-derived CO, and endothelial cells increase CO production in response to glutamate, a cerebral vasodilator (25, 26). Authentic CO or HO substrates dilate vascular preparations from anatomically diverse locations, including cerebral, coronary, pulmonary, and renal arteries and the aorta and ductus arteriosus (for reviews, see Refs. 30, 44, and 50). Vasodilation to heme, but not CO, is blocked by HO inhibitors (e.g., see Ref. 28), and reducing HO-2 expression increases vasoconstrictor sensitivity, an effect that is reversed with exogenous CO (20). In adult smooth muscle cell preparations, several intracellular signaling pathways have been proposed to explain the dilatory actions of CO, including activation of soluble guanylyl cyclase (45), cGMP-independent mechanisms (45), and inhibition of vasoconstrictor synthesis (39). One consistent finding is that inhibitors of large-conductance Ca\textsuperscript{2+}-activated K\textsubscript{+} (K\textsubscript{Ca}) channels block dilations to CO (20, 45, 49). CO activates K\textsubscript{Ca} channels not only in intact adult smooth muscle cells but also in excised membrane patches (46, 47), suggesting that CO may act directly on channel proteins.

CO is also a potent vasodilator in the newborn cerebral circulation, and dilations of pial arterioles to CO are prevented by K\textsubscript{Ca} channel blockers (28). CO activates K\textsubscript{Ca} channels in newborn cerebral artery smooth muscle cells, leading to an increase in effective coupling to localized intracellular Ca\textsuperscript{2+} transients termed “Ca\textsuperscript{2+} sparks" (16). This enhanced coupling ultimately leads to vasodilation via a decrease in global intracellular Ca\textsuperscript{2+} concentration (16). However, signaling pathways that lead to K\textsubscript{Ca} channel activation by CO in newborn cerebral arterial smooth muscle cells are unknown. Conceivably, CO may activate newborn K\textsubscript{Ca} channels via several mechanisms, similar to those that have been proposed to occur in the adult.

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Investigating the regulation of newborn cerebral arteriole smooth muscle cell KCa channels by CO will not only improve our knowledge of mechanisms that regulate the newborn cerebral vasculature but should also advance our understanding of mechanisms that enhance coupling of KCa channels to intracellular Ca$^{2+}$ signals.

The goal of this study was to investigate signaling mechanisms that underlie CO activation of KCa channels in newborn cerebral arteriole smooth muscle cells. Data show that CO or CO released from dimanganese decacarbonyl (DMDC), a novel CO donor, directly activates KCa channels in intact cells. In excised patches, newborn KCa channels exhibited low Ca$^{2+}$ sensitivity, a property that could be explained by reduced expression of the auxiliary $\beta_1$-subunit when compared with the $\alpha$-subunit. CO decreased the apparent dissociation constant ($K_d$) for Ca$^{2+}$ via a direct effect on the KCa channel and increased activity most effectively at micromolar when compared with nanomolar Ca$^{2+}$ concentrations. In addition, CO left shifted the current-voltage (I-V) relationship of cso-$\alpha$ currents expressed in HEK-293 cells. These data suggest that CO activates low-affinity KCa channels in newborn cerebral arteriole smooth muscle cells via a direct effect on the $\alpha$-subunit.

MATERIALS AND METHODS

Tissue preparation. All procedures that involve animals were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center.

Newborn pigs (1–3 days old, 1.25 kg) were anesthetized with ketamine hydrochloride (33 mg/kg im) and acepromazine (3.3 mg/kg im). The brain was removed and maintained in ice-cold HEPES-buffered physiological saline solution (PSS) containing (in mM) 134 NaCl, 6 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 10 glucose (pH 7.4, NaOH). Isolated arterioles (50–200 $\mu$m) were dissected from the brain and cleaned of basolateral connective tissue. Individual smooth muscle cells were dissociated from cerebral arterioles by using a procedure described previously (15).

Measurement of CO production by DMDC. DMDC dissolved in Krebs solution was placed inside clear glass vials (2.0 ml). The internal standard (see below) was injected into the bottom of the vial, and the vial was immediately sealed with a rubberized Teflon-lined cap. Vials were incubated at 37°C in bright light with the exception of the control, which was kept in darkness, and a sample of the headspace gas was injected into the gas chromatograph after 10 min. A saturated solution of the isotopically labeled CO (13$\ C^{16}$O) (isotopic purity $\geq$99%) was used as an internal standard for quantitative measurements by gas chromatography-mass spectrometry analysis of the headspace gas was performed using a Hewlett-Packard 5970A mass-selective ion detector interfaced to a Hewlett-Packard 5890A gas chromatograph. The separation of CO from other gases was carried out on a Varian-5A molybdenum capillary column (30 m; 0.32 mm ID) with a linear temperature gradient from 35°C to 65°C at 5°C per minute. Helium was the carrier gas at a column head pressure of 4.0 psi. Aliquots (100 $\mu$l) of the headspace gas were injected by using a gas-tight syringe into the spittleless injector having a temperature of 120°C. Ions at a mass-to-charge ratio (m/z) 28 and 29, corresponding to $^{12}$C$^{16}$O and $^{13}$C$^{16}$O, respectively, were recorded via selective ion monitoring. The amount of CO in samples was calculated from the ratio of peak areas m/z 28 and m/z 29.

Solutions containing 100 nM, 1 $\mu$M, and 10 $\mu$M DMDC gave no responses above 100 nM, and 10 $\mu$M DMDC gave no responses below 800 nM. The detection limit of the gas chromatograph was 1 nM, 3 $\mu$M, and 30 $\mu$M, respectively. In the presence of light with no air space, CO concentrations of 400 nM, 3 $\mu$M, and 30 $\mu$M, respectively, would be produced. The solution of 10 $\mu$M DMDC that was maintained in the dark did not produce detectable CO.

Expression of cso-$\alpha$ channels in HEK-293 cells. HEK-293 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM media (Mediatech; Herndon, VA) supplemented with 10% FBS (Sigma; St. Louis, MO), 1% penicillin-streptomycin, and amphotericin B (0.05%) in a 95% air-5% CO$_2$ humidified incubator at 37°C. Cells were subcultured once per week using trypsin-EDTA, after which cells were washed twice with culture media and replated on 35 mm petri dishes. Twenty-four hours after subculture, cso-$\alpha$ DNA (43) was transfected into the HEK cells by using the Effectene transfection kit (Qiagen; Valencia, CA). Briefly, cells were exposed to a preprepared mixture containing (amount per petri dish) 0.4 $\mu$g cso-$\alpha$ DNA, 0.05 $\mu$g enhanced green fluorescent protein DNA, 4 $\mu$l transfection enhancer, and 4 $\mu$l transfection reagent for 15–24 h, after which the cells were washed with fresh media. Electrophysiological recordings were performed 24–72 h after the transfection procedure. With the use of the patch-clamp setup, enhanced green fluorescent protein was excited at 450–490 nm, and emitted light between 500 and 550 nm was observed. Transfected cells were positively identified due to their emitted green fluorescence.

Control experiments were performed on cells (referred to as sham transfected) that were incubated with the same transfection mixture described above, but cso-$\alpha$ DNA was not included.

Patch-clamp electrophysiology. Potassium currents were measured in isolated smooth muscle cells or HEK-293 cells by using the perforated-patch, the conventional whole cell, or the inside-out configuration of the patch-clamp technique. For experiments performed using the perforated-patch and conventional whole cell configurations, the bathing solution contained (in mM) 134 NaCl, 6 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 10 glucose (pH 7.4, NaOH). The perforated-patch pipette solution contained (in mM) 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl$_2$, 10 HEPES, and 0.05 EGTA (pH 7.2 with KOH). Amphotericin B (1 $\mu$g/ml) was included in the pipette solution during perforated patch-clamp experiments. To obtain steady-state KCa channel measurements in perforated patch-clamp experiments, Ca$^{2+}$ sparks and thus transient KCa currents were abolished using thapsigargin (100 nM), an inhibitor of sarcoplasmic reticulum Ca$^{2+}$-ATPase (see Ref. 17). Membrane currents were recorded at 0 mV, filtered at 1 kHz using a low-pass Bessel filter, and digitized at 2.5 kHz. For inside-out recordings, the pipette solution contained (in mM) 140 NaCl, 6 KCl, 10 HEPES, 1 CaCl$_2$, and 2 MgCl$_2$. The inside-out bath solution contained (in mM) 140 KCl, 10 HEPES, 2 MgCl$_2$, 5 or 1 EGTA, 1.6 HEDTA, and appropriate amounts of CaCl$_2$ to obtain free Ca$^{2+}$ concentrations of 0.1, 0.3, 1, 3, 10, 30, or 100 $\mu$M. Free Ca$^{2+}$ concentrations in solutions were calculated by using Max Chelator Sliders software (C. Patton, Stanford University) and measured with a Ca$^{2+}$-selective and reference electrode (Corning; Acton, MA). Single channel currents in excised inside-out patches were recorded at 0 mV, filtered at 2.5 kHz, and digitized at 10 kHz.

Expressed cso-$\alpha$ currents were measured in HEK-293 cells using the conventional whole cell configuration. The conventional whole cell pipette solution contained (in mM) 30 KCl, 110 potassium aspartate, 10 NaCl, 0.3 CaCl$_2$, 1 MgCl$_2$, 1 MgATP, 0.1 Na-GTP, 10 HEPES, and 1 EGTA. The free Ca$^{2+}$ and Mg$^{2+}$ concentrations of this solution are 100 nM and 1 mM, respectively. Cso-$\alpha$ currents were activated from a holding potential of −70 mV to test potentials between −50 and +80 mV for 200 ms in 10-mV increments every 5 s. Membrane currents were filtered at 1 kHz and digitized at 4 kHz.

For single channel analysis, KCa channel activity ($N_p$) was calculated from continuous gap-free data by using Fetchan 6 (Axon Instruments, Union City, CA). The mean times of analysis under each condition for the entire study are as follows: control, 4.1 ± 0.1 min (n = 61); CO, 5.3 ± 0.5 min (n = 15); and DMDC, 4.1 ± 0.2 min (n = 39). For perforated-patch recordings, $N_p$ was calculated from.
the following equation: \( N_{P_o} = (\sum \tau_i) / T \), where \( \tau_i \) is the open time for each level \( i \) and \( T \) is the total time of analysis. For inside-out recordings, open probability (\( P_o \)) was calculated from the following equation: \( P_o = N_{P_o} / n \), where \( n \) is the total number of channels in the patch (determined by application of 100 \( \mu \)M free \( Ca^{2+} \) at 0 mV). Where appropriate, data were fit with a Hill equation: 
\[ P_o = P_{max} [Ca^{2+}]^{nH} / (K_{Ca}^{nH} + [Ca^{2+}]) \]
where \( nH \) is the Hill coefficient, \( P_{max} \) is maximal \( P_o \), and \( [Ca^{2+}] \) is the free \( Ca^{2+} \) concentration.

**Western blot analysis.** Rat (middle cerebral) and pig cerebral arteries (200 \( \mu \)m in diameter) were dissected from the brain, cleaned of connective tissue, and minced on ice. Total tissue lysates were prepared by using Laemmli sample buffer containing 2.5% SDS, 10% glycerol, 0.01% bromphenol blue, and 5% (vol/vol) \( \beta \)-mercaptoethanol in 100 mM Tris-HCl (pH 6.8) (10 min at 100°C). To measure protein concentration in the samples, 5-\( \mu \)l lysate aliquots were dotted on a nitrocellulose membrane, which were then washed with amido black solution, and the protein amount was then quantified spectrophotometrically at 630 nm. Proteins (25 \( \mu \)g/lane) were separated by 12% SDS-polyacrylamide gel electrophoresis (25 mA/gel) and electrotransferred to Hybond-P membranes (Amersham Biosciences; Piscataway, NJ) in a buffer containing 25 mM Tris, 192 mM glycine, 0.1% (wt/vol) SDS, and 20% (vol/vol) methanol (1 h at 350 mA). To block nonspecific binding sites, membranes were incubated in phosphate-buffered saline-0.1% Tween-20 (PBS-T) containing 5% (vol/vol) BSA overnight at +4°C. To detect \( KCa \) \( \alpha \)-subunit expression, membranes were incubated for 2 h at room temperature with rabbit polyclonal antibody to a COOH-terminal peptide (amino acids 1098–1196) of the mouse Slo (mSlo, at 1:2,000 dilution in PBS-T with 1% BSA; Alomone; Jerusalem, Israel). Blots were washed five times with PBS-T and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (dilution 1:100,000 in PBS-T with 1% BSA, Sigma). To detect \( KCa \) \( \beta \)-subunit expression, membranes were probed with goat polyclonal antibodies raised against a peptide mapping within the human \( KCa \) \( \beta_1 \)-subunit (amino acids 100–150, Y-17 from Santa Cruz Biotechnology, at 1:2,000 dilution) followed by HRP-conjugated anti-goat IgG (1:10,000 final dilution in PBS-T, Sigma). To determine specificity of the polyclonal antibodies, control blocking experiments were performed by using antibody preadsorbed with the corresponding antigenic peptide (antigen-to-antibody ratio, 4:1, wt/wt). To normalize antigen expression to a major housekeeping gene, corresponding antigenic peptide (antigen-to-antibody ratio, 4:1, wt/wt). To normalize antigen expression to a major housekeeping gene, membranes were reprobed with monoclonal antibodies against a highly conserved region of actin (1:10,000 dilution, Roche Molecular Biochemicals; Indianapolis, IN) followed by HRP-conjugated anti-mouse IgG (1:20,000 dilution, Jackson Immunoresearch; West Grove, PA). To visualize the bands, blots were incubated with the Western lighting chemiluminescence reagents Plus (Perkin-Elmer Life Sciences; Boston, MA) according to the manufacturer’s protocol and exposed to X-ray film (Hyperfilm, Amersham Pharmacia Biotech; Piscataway, NJ) in the linear response range. Band intensity was quantified by digital densitometry using National Institutes of Health Image version 1.63 software.

**Statistical analysis.** Values are expressed as means ± SE. Student’s \( t \)-test or repeated measures ANOVA with Tukey’s test was used for comparing paired or multiple data sets, respectively. \( P < 0.05 \) was considered significant.

**Chemicals.** Unless stated otherwise, all chemicals used in this study were obtained from Sigma Chemical. Papain was purchased from Worthington Biochemical (Lakeewood, NJ). Paxilline was purchased from AG Scientific (San Diego, CA). Desiccated DMDC was stored in the dark under nitrogen to reduce molecular decomposition. DMDC solutions were placed in a sealed container and exposed to light for 10 min before addition to the experimental chamber. When applied to cells or excised patches, the DMDC solution within the experimental chamber was exposed to the 30-W light of the inverted microscope to accelerate decomposition and CO release. Where applicable, an aqueous solution of DMDC was inactivated by exposure to room light for 24–72 h.

**RESULTS**

**CO or CO released from DMDC, activates \( KCa \) channels in newborn cerebral arteriole smooth muscle cells.** Isolated cells were voltage clamped at 0 mV by using the perforated-patch configuration of the patch-clamp technique. \( KCa \) channel activity was measured in the control and after bath application of CO or DMDC, a novel light-activated CO donor (32). Openings of large-conductance \( KCa \) channels were identified based on the characteristic single channel conductance and block by tetraethylammonium as we previously described (16). CO (3 \( \mu \)M) or DMDC (10 \( \mu \)M), when exposed to light, reversibly increased \( N_{P_o} \) by ~4.9-fold or ~3.5-fold, respectively (Fig. 1). A
10 μM DMDC solution exposed to light for 10 min should produce ~3 μM CO (see MATERIALS AND METHODS). This would explain the similar elevation in K<sub>Ca</sub> channel activity by 3 μM CO and 10 μM DMDC. In the absence of light, DMDC did not produce any detectible CO or alter K<sub>Ca</sub> channel activity (see MATERIALS AND METHODS and Fig. 1). The data indicate that purified dissolved CO gas, or CO released from DMDC, activates K<sub>Ca</sub> channels in newborn porcine cerebral arteriole smooth muscle cells. These data also identify DMDC as a useful tool for studying CO regulation of K<sub>Ca</sub> channels.

**CO activates K<sub>Ca</sub> channels in newborn cerebral arteriole smooth muscle cells via a soluble guanylyl cyclase-independent mechanism.** Although CO is a poor activator of soluble guanylyl cyclase (24), in some vascular preparations, dilations induced by CO have been attributed to this mechanism (7). To investigate signaling mechanisms that lead to K<sub>Ca</sub> channel activation by CO, DMDC was applied to voltage-clamped (perforated patch, 0 mV) cells in the presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a soluble guanylyl cyclase inhibitor. ODQ (1 μM) blocked dilations to sodium nitroprusside (10 μM), a nitric oxide (NO) donor, in isolated pressurized (30 mmHg) newborn cerebral arterioles, confirming the ability to inhibit soluble guanylyl cyclase (data not shown, but see also Ref. 23 for similar findings). The same ODQ solution (at 25 μM) did not significantly change mean K<sub>Ca</sub> channel activity in voltage-clamped cells (Fig. 2). When applied in the continued presence of ODQ in the same membrane patches, CO (3 μM) increased mean K<sub>Ca</sub> channel activity 6.9-fold (Fig. 2). These data suggest that CO activates K<sub>Ca</sub> channels in newborn arteriole smooth muscle cells via a mechanism that does not involve soluble guanylyl cyclase. CO released from DMDC activates K<sub>Ca</sub> channels in excised inside-out membrane patches of newborn arteriole smooth muscle cells. A: original current recordings from the same patch illustrating K<sub>Ca</sub> channel activation by DMDC (1 μM) and washout. Free Ca<sup>2+</sup> concentration in the bathing solution was 3 μM. C, closed channel level, i.e., zero current. B: average effects of DMDC (n = 9) or light-inactivated DMDC (inact., n = 5) on K<sub>Ca</sub> channel P<sub>o</sub> in inside-out patches. *P < 0.05.

**Fig. 3.** CO released from DMDC activates K<sub>Ca</sub> channels in excised inside-out membrane patches of newborn arteriole smooth muscle cells. A: original current recordings from the same patch illustrating K<sub>Ca</sub> channel activation by DMDC (1 μM) and washout. Free Ca<sup>2+</sup> concentration in the bathing solution was 3 μM. C, closed channel level, i.e., zero current. B: average effects of DMDC (n = 9) or light-inactivated DMDC (inact., n = 5) on K<sub>Ca</sub> channel P<sub>o</sub> in inside-out patches. *P < 0.05.

**CO activates K<sub>Ca</sub> channels in excised membrane patches.** Because K<sub>Ca</sub> channel activation by CO was not caused by activation of soluble guanylyl cyclase, we sought to investigate whether CO can activate K<sub>Ca</sub> channels in the complete absence of intracellular signaling factors. CO regulation of K<sub>Ca</sub> channel activity was measured in excised inside-out membrane patches with 3 μM free Ca<sup>2+</sup> present in the bath solution. At 0 mV, DMDC (1 μM) increased mean K<sub>Ca</sub> channel P<sub>o</sub> ~3.1-fold. In contrast, light-inactivated DMDC (1 μM) did not alter K<sub>Ca</sub> channel activity (Fig. 3). These data suggest that CO released from DMDC activates K<sub>Ca</sub> channels in excised membrane patches from newborn cerebral arteriole smooth muscle cells.

**CO increases the apparent Ca<sup>2+</sup> sensitivity of newborn arteriole smooth muscle cell K<sub>Ca</sub> channels.** K<sub>Ca</sub> channel activity is regulated by membrane voltage and intracellular Ca<sup>2+</sup> concentration (for review see Ref. 34). Conceivably, CO could activate K<sub>Ca</sub> channels by increasing apparent Ca<sup>2+</sup> sensitivity. To determine the Ca<sup>2+</sup> sensitivity of K<sub>Ca</sub> channels in newborn arteriole smooth muscle cells, P<sub>o</sub> was measured in inside-out membrane patches from newborn arteriole smooth muscle cells over a wide range of intracellular free Ca<sup>2+</sup> concentrations from 300 nM to 100 μM.

The mean apparent K<sub>d</sub> for Ca<sup>2+</sup> at 0 mV was 31.3 ± 0.4 μM with an n<sub>H</sub> of 3.1 ± 0.2 and a maximum P<sub>o</sub> of 0.81 ± 0.04 (n = 6, Fig. 4, A and B). In the same membrane patches, DMDC (1 μM) decreased the mean K<sub>d</sub> for Ca<sup>2+</sup> of K<sub>Ca</sub> channels to 24 ± 2.8 μM but did not alter the n<sub>H</sub> or the...
Data were fit with a Hill equation. In control, the mean apparent dissociation constant ($K_o$) for Ca$^{2+}$ at 0 mV was 31.3 ± 0.4 μM with a Hill coefficient of 3.1 ± 0.2 and a maximum $P_o$ of 0.81 ± 0.04 (n = 6). In the same patches, DMDC (1 μM) decreased the mean $K_o$ for Ca$^{2+}$ of KCa channels to 24.2 ± 2.8 μM but did not alter the Hill coefficient (3.1 ± 0.2) or the maximum $P_o$ (0.84 ± 0.05). C: average relative increase in $P_o$ induced by DMDC over a range of free Ca$^{2+}$ concentrations. In 300 nM, 1 μM, 3 μM, and 10 μM Ca$^{2+}$, DMDC (1 μM) increased KCa channel $P_o$ 1.1 ± 0.1-fold, 1.4 ± 0.4-fold, 1.9 ± 0.5-fold, and 3.7 ± 0.7-fold, respectively.

maximal $P_o$ (Fig. 4, A and B). Although DMDC increased KCa channel $P_o$ at all Ca$^{2+}$ concentrations, relative activation by DMDC increased dramatically between 300 nM and 10 μM Ca$^{2+}$ (Fig. 4C). For example, with 300 nM free Ca$^{2+}$ present at the cytosolic surface of KCa channels, DMDC increased KCa $P_o$ 1.1-fold (i.e., 10%), whereas with 10 μM free Ca$^{2+}$ DMDC increased $P_o$ 3.7-fold (i.e., 270%, Fig. 4C). It could not be determined whether CO was more effective at activating KCa channels at Ca$^{2+}$ concentrations >10 μM Ca$^{2+}$. With 30 and 100 μM Ca$^{2+}$, $P_o$ was already high (0.53 and 0.86, respectively), precluding further direct comparison of any relative increase.

These data suggest that CO activates KCa channels in excised membrane patches of newborn arteriole smooth muscle cells by increasing apparent Ca$^{2+}$ sensitivity. Data also suggest that CO is a particularly effective activator of KCa channels exposed to low micromolar Ca$^{2+}$ concentrations.

CO activates cslo-α subunits expressed in HEK-293 cells. In smooth muscle cells, KCa channels can be composed of Ca$^{2+}$- and voltage-sensitive pore-forming α-subunits and auxiliary β1-subunits that increase apparent Ca$^{2+}$ sensitivity (4). Because CO increases the apparent Ca$^{2+}$ sensitivity of KCa channels in newborn arteriole smooth muscle cells, we sought to determine whether this effect was dependent on the presence of a β-subunit. To address this question, we studied CO regulation of KCa channel α-subunits, which are expressed in arterial smooth muscle and can be activated via PKG-mediated phosphorylation (11, 43). Cslo-α subunit DNA was transfected into HEK-293 cells, which do not express endogenous β-subunits (12). With the use of the conventional whole cell configuration, cslo-α currents were activated from a holding potential of −70 mV to test potentials between −50 and +80 mV in 10-mV increments.

In sham-transfected HEK-293 cells, depolarization-induced outward currents were small (mean current at +80 mV: control, 173 ± 83 pA), unaffected by DMDC (1 μM, 160 ± 78 pA), and insensitive to paxilline, a selective KCa channel blocker (Ref. 40, 100 nM, 152 ± 82 pA at +80 mV, n = 5). Transient transfection of cslo-α subunit DNA resulted in the appearance of large depolarization-induced outward currents (Fig. 5A). DMDC (1 μM) activated cslo-α currents at voltages positive to −30 mV and produced a parallel leftward shift in the I-V relationship (n = 9, Fig. 5, A and B), consistent with an increase in the apparent Ca$^{2+}$ sensitivity of the cslo-α channel. For example, DMDC increased mean cslo-α currents 2.2-fold at +50 mV. Paxilline (100 nM) reduced DMDC-activated currents to an amplitude similar to that of sham-transfected cells, indicating that DMDC activated expressed cslo-α channels (n = 4, Fig. 5, A and B). These data suggest that CO activates cslo-α channels expressed in HEK-293 cells.

Western immunoblotting of KCa channel α- and β-subunits. The Ca$^{2+}$ sensitivity of the newborn arteriole smooth muscle cell KCa channel described in this study (Fig. 4) is similar to that of the α-subunit when expressed in the absence of an auxiliary β-subunit (31). To determine whether the low Ca$^{2+}$ sensitivity is due to the absence of an expressed β-subunit, we detected KCa channel α- and β-subunits in cerebral arterioles of newborn pigs. The Ca$^{2+}$ sensitivity of the adult rat arterial smooth muscle KCa channel is well described and similar to that of an associated α- and β-subunit (4, 31, 35). To provide a standard with which to compare our data, we also detected protein expression of KCa channel α- and β-subunits in adult rat cerebral arteries and aorta.

Immunoblotting for the KCa channel α-subunit with antibodies raised against a COOH-terminal peptide of mSlo recognized a major protein band of ~125 kDa in rat and pig cerebral arteries and rat aorta (Fig. 6A). This band corresponds to the molecular mass of the α-subunit detected in vascular smooth muscle from different animal species (21). The amino acid sequence of the epitope recognized by the antibody is 95% identical between rat and pig KCa channels (accession numbers...
AAP82454 and AF026000). In addition to a major 125-kDa band, a minor band with lower molecular mass of \( \sim 60 \) kDa was detected (not shown) that corresponds to a proteolytic fragment of the \( \alpha \)-subunit (21). Antibody specificity was confirmed by preadsorption of the antibody with an antigenic peptide, which abolished the bands seen at 125 and 60 kDa. Western blot analysis for the \( \beta \)-subunit performed by using polyclonal antibodies directed against human \( \kappa_{Ca} \beta \) (Y-17, Santa Cruz Biotechnology) revealed a single immunoreactive protein band with a molecular mass of \( \sim 30 \) kDa. This band corresponds to the \( \kappa_{Ca} \) channel \( \beta \)-subunit in bovine aorta and tracheal smooth muscle (Fig. 6A) (22). Specificity for the \( \kappa_{Ca} \) channel \( \beta \)-subunit was confirmed by preadsorption of the antibody with an antigenic peptide, which abolished the immunoreactive band. The amino acid sequence of the epitope recognized by the antibody (amino acids 100–150) is highly conserved among rat, mouse, human, and bovine \( \kappa_{Ca} \) channel \( \beta \)-subunits, exhibiting \( \sim 80\% \) homology (see Ref. 18 for sequence alignment). With the assumption that the antibodies recognize the pig and rat epitopes with equal affinity, \( \alpha \)-subunit expression normalized to actin was \( \sim 1.5\)-fold greater and \( \beta \)-subunit expression was \( \sim 2\)-fold lower in newborn pig cerebral arteries when compared with rat (Fig. 6, B and C). Thus the expression ratio of \( \beta \)-subunit-to-\( \alpha \)-subunits in newborn pig cerebral arteries appears to be \( \sim 2\)- to 3-fold lower than that in adult rat (\( P < 0.05 \)).

**DISCUSSION**

This study presents the following novel findings: 1) dissolved, gaseous CO or DMDC (a novel CO donor) activates \( \kappa_{Ca} \) channels in newborn cerebral arteriole smooth muscle cells via a soluble guanylyl cyclase-independent mechanism; 2) DMDC is a useful tool for studying CO regulation of \( \kappa_{Ca} \) channels because nonspecific effects of this compound were not observed; 3) newborn \( \kappa_{Ca} \) channels exhibit low \( \Ca^{2+} \) sensitivity, a property that could be explained due to decreased expression of \( \beta \)-relative to \( \alpha \)-subunits; 4) CO directly activates newborn \( \kappa_{Ca} \) channels by increasing the apparent \( \Ca^{2+} \) sensitivity; and 5) CO is most effective at activating \( \kappa_{Ca} \) channels exposed to micromolar when compared with nanomolar, free \( \Ca^{2+} \) concentrations, which should lead to preferential activation by localized signaling modalities that elevate the intracellular \( \Ca^{2+} \) concentration within this range. In summary, data from the present study suggest that in newborn arteriole smooth muscle cells, CO activates low-affinity \( \kappa_{Ca} \) channels...
via a direct molecular interaction with the α-subunit that increases apparent Ca\(^{2+}\) sensitivity.

**CO increases \(K_{Ca}\) channel apparent Ca\(^{2+}\) sensitivity.** CO is a potent dilator of the newborn cerebral vasculature (16, 28). CO activates \(K_{Ca}\) channels in intact newborn cerebral arteriole smooth muscle cells, and dilations to CO are blocked by inhibitors of \(K_{Ca}\) channels (16). However, signal transduction mechanisms that lead to the activation of \(K_{Ca}\) channels by CO in newborn arteriole smooth muscle cells are unknown. In the present study, DMDC and CO activated \(K_{Ca}\) channels in intact cells and in excised membrane patches. DMDC is a novel CO donor that relaxes arterial preparations, but the regulation of ion channels by DMDC has not been investigated (9, 32). Data presented here indicate that DMDC is a useful tool for studying CO regulation of \(K_{Ca}\) channels. Light-inactivated DMDC, or DMDC applied in the dark, did not alter \(K_{Ca}\) channel activity in intact cells or excised patches, indicating that DMDC does not induce nonspecific activation of \(K_{Ca}\) channels.

In excised patches, CO shifted the Ca\(^{2+}\)-concentration-response curve of \(K_{Ca}\) channels leftward, suggesting that activation occurs via an increase in apparent Ca\(^{2+}\) sensitivity. CO was most effective at activating \(K_{Ca}\) channels exposed to micromolar Ca\(^{2+}\) concentrations, increasing \(K_{Ca}\) channel \(P_{\alpha}\) 270% at 10 \(\mu M\) Ca\(^{2+}\) but only 10% at 300 \(nM\) Ca\(^{2+}\). CO also increased the apparent Ca\(^{2+}\) sensitivity of \(K_{Ca}\) channels in cultured adult rat tail artery smooth muscle cells but at higher CO concentrations than those used in the present study (46).

The CO concentration in cerebrovascular smooth muscle cells in vivo is not known. Recently, the CO concentration in cortical periarachnoid fluid (artificial cerebrospinal fluid, aCSF) from newborn pigs was measured (5). CO accumulation in aCSF placed under a cranial window for 10 min was 88 ± 20 pmol/ml (or ~0.1 \(\mu M\)). Therefore, in cells of the brain that generate CO, the minimal concentration would be 0.1 \(\mu M\). A gradient should exist between cells and the aCSF. CO concentrations in and near CO-producing cells, including microvascular cells, will be higher than in the cortical periarachnoid fluid (26). Furthermore, within the cell, heme metabolism should create local intracellular gradients that are higher than CO concentrations found globally. HO may be located near cellular targets, and CO concentrations produced near \(K_{Ca}\) channels may be higher than global cellular concentrations. Conceivably, in arterial smooth muscle cells in vivo, \(K_{Ca}\) channels may be exposed to 3 \(\mu M\) CO under basal conditions (i.e., \(30 \times\) that found in aCSF) and much higher concentrations if CO production were stimulated.

**Molecular composition of newborn cerebral \(K_{Ca}\) channels.** In smooth muscle, \(K_{Ca}\) channels can be composed of Ca\(^{2+}\)- and voltage-sensitive, pore-forming α-subunits and an auxiliary β\(_1\)-subunit that increases Ca\(^{2+}\) sensitivity (4, 35, 41). The Ca\(^{2+}\) sensitivity of \(K_{Ca}\) channels in human coronary artery smooth muscle cells is 4 \(\mu M\) at 0 mV (41), and in adult rat arterial smooth muscle cells Ca\(^{2+}\) sensitivity is 19 \(\mu M\) at -40 mV (35). In comparison, the \(K_{Ca}\) for Ca\(^{2+}\) of newborn arteriole smooth muscle cell \(K_{Ca}\) channels was 31 \(\mu M\) at 0 mV, which is similar to the Ca\(^{2+}\) sensitivity of α-subunits when expressed in the absence of auxiliary β-subunits (31). To investigate whether the low Ca\(^{2+}\) sensitivity of newborn cerebral arteriole smooth muscle \(K_{Ca}\) channels may be due to reduced expression of α-subunit, we quantified subunit expression. Western immunoblotting suggests that relative expression of β- to α-subunits is ~3-fold lower in cerebral arterioles from newborn pigs when compared with that of adult rat cerebral arteries and the aorta. These quantitative differences are unlikely to be due to species-specific differences in antibody affinities. Antibodies used to detect α-subunit expression were directed against a COOH-terminal peptide that is 95% identical between rat and pig \(K_{Ca}\) channels (accession numbers AAP82454 and AF026000). Similarly, the amino acid sequence of the β-subunit epitope recognized by the antibody is highly conserved among different species (18). It has been estimated that tracheal smooth muscle \(K_{Ca}\) channels exhibit a 1α/1β-subunit stoichiometry (13). Our data suggest that in newborn pig cerebral arteriole smooth muscle cells, free pore-forming α-subunits may account for the reduced \(K_{Ca}\) channel Ca\(^{2+}\) sensitivity. In the fetal brain, the neuron-specific \(K_{Ca}\) channel β4-subunit is expressed to a much lesser extent (5- to 10-fold) than in the adult (22). Conceivably, expression of \(K_{Ca}\) channel β-subunits may be developmentally regulated in many tissues. Data also suggest that in adult rat arterial smooth muscle, expression of the β-subunit may be greater than that of the α-subunit. Conceivably, this may be necessary to induce the higher Ca\(^{2+}\) sensitivity. Whether the reduced β-to α-subunit ratio in the newborn entirely explains the low Ca\(^{2+}\) sensitivity cannot be concluded from our data. Other potential explanations for low \(K_{Ca}\) channel Ca\(^{2+}\) sensitivity in the newborn may include the expression of an α- or β-subunit splice variant that differs from the adult (e.g., see Ref. 19) or an additional membrane-bound \(K_{Ca}\) channel modulator that reduces Ca\(^{2+}\) sensitivity.

**CO activates cslo-α-subunits.** NO and CO can activate \(K_{Ca}\) channels directly (3, 46). NO also activates soluble guanylyl cyclase, and ultimately increases \(K_{Ca}\) channel activity via PKG-mediated phosphorylation (1, 10, 11, 37). In contrast, CO is a poor activator of soluble guanylyl cyclase (24). Data in the present study suggest that CO does not activate \(K_{Ca}\) channels in newborn arteriole smooth muscle cells via soluble guanylyl cyclase. Conceivably, CO activation of \(K_{Ca}\) channels could occur via a direct interaction with the α-subunit or with an associated regulatory molecule, such as heme or a heme protein (42). Although CO activates \(K_{Ca}\) channels that are removed from the intracellular milieu, in intact cells CO could potentially be a more effective channel activator due to the presence of additional cytosolic signaling and permissive enabling factors such as NO (27).

A recent study demonstrated that CO activates mslo-α channels expressed in COS-1 cells (47). However, mslo-α channels and native arterial smooth muscle \(K_{Ca}\) channels do not appear to be similarly regulated. For example, NO does not activate mslo-α channels directly or via channel phosphorylation but via a direct effect on an auxiliary β-subunit (47). In contrast, NO activates native \(K_{Ca}\) channels via PKG-mediated phosphorylation (10, 37). In the present study, to investigate the \(K_{Ca}\) channel subunit requirements for CO activation, the regulation of cslo-α currents was studied. Cslo-α subunits are expressed in arterial smooth muscle (43), and NO can activate cslo-α subunits via PKG-mediated phosphorylation (11). Cslo-α currents activated by step depolarization showed a similar I-V relationship and activation kinetics to those described previously (11, 12, 43). In intact cells, CO shifted the I-V relationship of cslo-α currents leftward, increasing currents 2.2-fold at +50 mV. Data therefore indicate that CO activates...
K\textsubscript{Ca} channel \(\alpha\)-subunits that are similar to those expressed in native arterial smooth muscle.

Physiological relevance of a CO-induced increase in \(K_{\text{Ca}}\) channel \(Ca^{2+}\) sensitivity. Recent evidence suggests that CO dilates newborn cerebral arterioles by increasing the effective coupling of \(Ca^{2+}\) sparks to \(K_{\text{Ca}}\) channels in smooth muscle cells (16). \(Ca^{2+}\) sparks are spatially restricted micromolar elevations in intracellular \(Ca^{2+}\) concentration that occur due to the opening of a number of ryanodine-sensitive \(Ca^{2+}\) release channels on the sarcoplasmic reticulum (17). In smooth muscle cells, \(Ca^{2+}\) sparks activate several nearby sarcocelometric \(K_{\text{Ca}}\) channels to induce a transient outward \(K_{\text{Ca}}\) current. In cerebral artery smooth muscle cells of adult rats and mice, essentially 100% of \(Ca^{2+}\) sparks evoke a transient \(K_{\text{Ca}}\) current (4, 6, 36). However, in newborn porcine cerebral arteriole smooth muscle cells, only \(\sim60\%\) of \(Ca^{2+}\) sparks induce a transient \(K_{\text{Ca}}\) current (16). \(K_{\text{Ca}}\) channels located nearby \(Ca^{2+}\) spark sites are typically exposed to between 4 and 30 \(\muM\) \(Ca^{2+}\) at the peak of a spark (35, 51). Conceivably, uncoupling occurs in newborn arteriole smooth muscle cells because \(K_{\text{Ca}}\) channels are less sensitive to \(Ca^{2+}\) sparks (see Fig. 4). Supporting this hypothesis is the observation that the mean \(Ca^{2+}\) spark amplitude in newborn arteriole smooth muscle cells is similar to that in other preparations where coupling is unitary (17). Furthermore, in the newborn, \(K_{\text{Ca}}\) channels are activated by larger amplitude sparks but are insensitive to smaller amplitude \(Ca^{2+}\) sparks (16), suggesting that uncoupling occurs at the level of the \(K_{\text{Ca}}\) channel.

Data from the present study suggest that CO is a particularly effective activator of \(K_{\text{Ca}}\) channels exposed to micromolar \(Ca^{2+}\) (35, 51). The large CO-induced increase in \(K_{\text{Ca}}\) channel activity at micromolar \(Ca^{2+}\) concentrations would significantly enhance effective coupling to \(Ca^{2+}\) sparks, leading to increase in transient \(K_{\text{Ca}}\) current frequency and amplitude (16). The ensuing membrane hyperpolarization would result in a decrease in voltage-dependent \(Ca^{2+}\) channel activity, a reduction in intracellular \(Ca^{2+}\) concentration, and vasodilatation (16, 17). Previous data from our group show that vasodilatation to CO is blocked by ryanodine, a ryanodine-sensitive \(Ca^{2+}\) release channel blocker that inhibits \(Ca^{2+}\) sparks (16). These data suggest that CO activation of \(K_{\text{Ca}}\) channels may be physiologically relevant only in the presence of \(Ca^{2+}\) sparks.

In summary, data suggest that in newborn cerebral arteriole smooth muscle cells, \(K_{\text{Ca}}\) channels are of low \(Ca^{2+}\) sensitivity, and CO activates \(K_{\text{Ca}}\) channels via a direct effect on the \(\alpha\)-subunit that leads to an increase in apparent \(Ca^{2+}\) sensitivity. Data also suggest that CO preferentially increases the micromolar \(Ca^{2+}\) sensitivity of \(K_{\text{Ca}}\) channels. This effect will enhance coupling to \(Ca^{2+}\) signaling modalities such as \(Ca^{2+}\) sparks that elevate the sub sarcocelommal \(Ca^{2+}\) concentration within this range.

ACKNOWLEDGMENTS

We thank R. Appling for gas chromatography/mass spectrometry measurements, Dr. Z. Fan for the enhanced green fluorescent protein DNA, and Drs. Cheranov and Dopico for helpful comments on the paper.

GRANTS

This study was supported by National Institutes of Health Grants NS-67061 (to J. H. Jaggar), HL-034059, and HL-042851 (to C. W. Leffler) and a grant from the American Heart Association National Center (to J. H. Jaggar).

REFERENCES


