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Chronic carbon monoxide enhanced IbTx-sensitive currents in rat resistance pulmonary artery smooth muscle cells

ERIC DUBUIS, MATHIEU GAUTIER, ALEXANDRE MELIN, MANUEL REBOCHO, CATHERINE GIRARDIN, PIERRE BONNET, AND CHRISTOPHE VANDIER
Laboratoire de Physiopathologie de la Paroi Artérielle, Faculté de Médecine, 37032 Tours, France

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Dubuis, Eric, Mathieu Gautier, Alexandre Melin, Manuel Rebocho, Catherine Girardin, Pierre Bonnet, and Christophe Vandier. Chronic carbon monoxide enhanced IbTx-sensitive currents in rat resistance pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 283: L120–L129, 2002. First published February 15, 2002; 10.1152/ajplung.00004.2002.—Exogenous carbon monoxide (CO) can induce pulmonary vasodilation by acting directly on pulmonary artery (PA) smooth muscle cells. We investigated the contribution of K^+ channels to the regulation of resistance PA resting membrane potential on control (PAC) rats and rats exposed to CO for 3 wk at 530 parts/million, labeled as PACO rats. Whole cell patch-clamp experiments revealed that the resting membrane potential of PACO cells was more negative than that of PAC cells. This was associated with a decrease of membrane resistance in PACO cells. Additional analysis showed that outward current density in PACO cells was higher (50% at +60 mV) than in PAC cells. This was linked to an increase of iberiotoxin (IbTx)-sensitive current. Chronic CO hyperpolarized membrane of pressurized PA from -46.9 ± 1.2 to -56.4 ± 2.6 mV. Additionally, IbTx significantly depolarized membrane of smooth muscle cells from PACO arteries but not from PAC arteries. The present study provides initial evidence of an increase of Ca^{2+}-activated K^+ current in smooth muscle cells from PA of rats exposed to chronic CO.

Kv; K channel blockers; 4-aminopyridine; pressurized artery; iberiotoxin

THE RELATIONSHIP BETWEEN MEMBRANE POTENTIAL (E_m) and tension in arterial smooth muscles has been well studied. It is accepted that tension in smooth muscle cells is, in part, regulated by the availability of Ca^{2+} in the cytosol, which depends in part on the activity of K^+ channels that control resting E_m. Pulmonary arterial (PA) smooth muscle cells have a high input resistance in the resting state [found to be 18 GΩ by Evans et al. (13) and 4.7 GΩ by Park et al. (29)] that corresponds to very low ionic channel activity near resting E_m. The consequence is that opening or closure of very few channels can cause a substantial change in E_m resulting in modification of arterial tone. On the other hand, PA smooth muscle cells have a high density of Ca^{2+}-activated K^+ (KCa) channels and voltage-activated K^+ (Kv) channels. Both of these channels regulate resting E_m of PA smooth muscle cells (3, 27, 37), and KCa is suspected to act as a feedback mechanism in regulation of Ca^{2+} entry (6); thus they are important in regulating PA tone (32). Carbon monoxide (CO) is an endogenously generated gas as well as a ubiquitous environmental pollutant. The two major sources of exogenous CO exposure are automotive emissions and cigarette smoke. Concentrations of CO found in urban environments have been correlated with hospital admissions, mortality, and morbidity caused by cardiovascular and pulmonary diseases (19, 24, 31, 41). Average CO concentrations have been found to be 1–9 parts per million (ppm), but there are many conditions in which exposures exceed these levels. Indeed, cigarette smoke contains CO levels ranging from 35 to 1,000 ppm (38), and exposure to high levels of CO can occur in numerous occupational settings, such as those experienced by tunnels workers (9). CO is also an endogenously generated gas that regulates vascular tone (39). The predominant biological source of CO is from degradation of heme by heme oxygenase (HO) (36). At least two isoforms of HO, HO-1 and HO-2, an inducible and a constitutive isoform, respectively, have been identified and are present in vascular smooth muscle cells (12). Most experiments suggest that endogenously generated CO affects vascular tone in physiological and pathological conditions, but these experiments were performed using an activator/blocker of HO to activate or inhibit the endogenous CO production (39). In PA smooth muscle cells, HO-1 is transiently increased by chronic hypoxia (7) whenever sustained activation prevents the development of hypoxic pulmonary hypertension (8). Nevertheless, there is no direct evidence that this last effect is due to CO, and an effect of chronic exogenous CO on pulmonary tone is lacking. However, several lines of investigation provide evidence that acute CO is a vasodilator acting directly on vascular smooth muscle cells (39) that also inhibits smooth muscle cell proliferation (23). In the pulmonary circulation, exogenous CO decreases vascular resistance (11) and inhibits hypoxic pulmonary vasoconstriction (34), probably by a direct vasorelaxing effect of CO on

Address for reprint requests and other correspondence: C. Vandier, Laboratoire de Physiopathologie de la Paroi Artérielle, Faculté de Médecine (LABPART), 2 bis Boulevard Tonnellé, 37032 Tours, France (E-mail: vandier@univ-tours.fr).

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smooth muscle cells (33). The mechanism of CO-induced PA relaxation is not known, but it could act by activating K^+ channels. Indeed, acute exposure to CO has been shown to activate KCa and Kv channels, respectively, in tail artery smooth muscle cells of rats (40) and in jujenal circular smooth muscle cells of humans (14). Nevertheless, the physiological and chronic importance of endogenous CO on K^+ channel functions was not directly studied. Indeed, only studies using blockers of HO to inhibit the endogenous CO production and then the changes in K^+ channel function have been performed (18, 21, 43).

It has been suggested that drug therapies acting on K^+ channel expression may have potential value for the treatment of vascular diseases (5). Because acute CO activates KCa channels and because these channels have been shown to be compensatory vasodilatory pathways in systemic hypertension (22), we speculated that chronic CO could activate these channels, which would explain, in part, the vasodilatory effect of CO in the pulmonary circulation.

In this study we examined for the first time the effect of chronic exogenous CO exposure on the regulation of resting E_m of pressurized resistance PA. Then we examined passive membrane properties and K^+ channel blocker-sensitive currents of resistance PA smooth muscle cells. The present study provides initial evidence of an increase of IbTx-sensitive K^+ current that hyperpolarizes resistance PA smooth muscle cells of rats exposed to chronic CO. A preliminary report of part of this study has been published in abstract form (10).

MATERIALS AND METHODS

Exposure to CO. All animal experiments were conducted according to the ethical standards of the Ministère Français de l'Agriculture for animal care and use. Exposure to CO was performed using methods previously described (4). Adult female Wistar rats (250 g) were placed in an exposure chamber inflated by an air-CO mixture for 3 wk at 530 ppm at 24°C (labeled as PACO). The sudden death of rats was avoided by gradually raising the CO concentration from 300 ppm on the first day to 400 ppm on the second day and 530 ppm on the third day. Another group of rats, the control group, was placed in the same chamber but without CO (labeled as PAC). During experiments, CO concentration in the exposure chamber was continuously monitored (Analyzer Surveyor S). The CO chamber was opened twice a week for <5 min for changing cages and replenishing them with food and water. Mean PA pressure was not significantly different between PAC and PACO rats (12.7 ± 1.2 vs. 13.3 ± 3.0 mmHg, $N = 3$).

Tissue preparation for microelectrode recording. Animals were deeply anesthetized with pentobarbital sodium (60 mg/kg ip). The thorax was opened, and lungs were rapidly excised and immersed in cold (4°C) physiological saline solution (PSS). In the left pulmonary lobe, an intrapulmonary resistance artery with an internal diameter of <150 μm was isolated and gently cleaned of parenchyma and adhering connective tissue under a dissection microscope. Collateral arterioles of the resistance artery were tied with 30-μm-thin silk to make surgical sutures. We removed the endothelium by passing an air bubble inside the artery.

PSS solution contained (in mM): 138.6 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 0.33 NaH_2PO_4, 10 HEPES, and 11 glucose. pH was adjusted to 7.4 using NaOH.

Recording of E_m on pressurized resistance arteries. A freshly prepared resistance artery was transferred in a 2.5-ml organ chamber hold at 37°C and filled with PSS. One end of the artery was mounted on a glass micropipette (Clark Electromedical Instrument) connected to a pressure captor (Baxter) and to a pressure controller (Hewlett Packard). The other end of the artery was mounted on a micropipette connected to a stopcock. The organ chamber was placed on the stage of a microscope (Olympus) fitted with a video camera (Sanyo) leading to a monitor (Sony). PSS at 37°C was used for both continuously superfusing (5 ml/min) and perfusing arteries. After 10 min of perfusion, the stopcock was closed and the intraluminal pressure was slowly increased to 14 mmHg. This level of pressure was maintained during a 60-min equilibration period before experiments.

E_m of arterial smooth muscle cells was recorded using glass microelectrodes (Clark Electromedical Instrument) filled with 3 M KCl solution and connected to a microelectrode amplifier (Biologic VF 180). The entire set-up was mounted on an antivibration system (TMC Micro-g), and manipulation was performed using a micromanipulator (Narishige). Response for K^+ channel blockers was recorded after adding aliquots of 4-aminopyridine (4-AP) for a final concentration of 3 mM in the chamber or iberiotoxin (IbTx, 100 nM) to block Kv channel type and KCa channel type, respectively. We added these drugs directly to the vessel chamber after stopping the superfusion.

Isolation of smooth muscle cells. Enzymatic isolation of single vascular smooth muscle cells was performed according to published methods of dissociation for rat microvessels (17). Briefly, intrapulmonary resistance arteries with internal diameters of <100 μm were placed in dissociation solution (Ca^{2+} free) for 10 min at room temperature. Tissue was then placed in dissociation solution at 37°C containing 1 mg/ml of papain (Sigma) and 1 mg/ml of dithioerythritol for 18 min. The vessel was then put in a dissociation solution at 37°C containing 1.6 mg/ml collagenase (type H, Sigma), 1.6 mg/ml trypsin inhibitor (type 1-S, Sigma), and 0.25 mg/ml elastase (type IV, Sigma) for 8 min. The tissue was then put in dissociation solution (free of enzymes) for 5 min and then gently agitated with a polished wide-bore Pasteur pipette to release the cells. Cells were stored at 4°C and used between 2 and 8 h after isolation. Only long, smooth, optically refractive cells were used for patch-clamp measurements.

Dissociation solution contained (in mM): 145 NaCl, 4 KCl, 1 $MgCl_2$, 10 HEPES, and 10 glucose. pH was adjusted to 7.3 using NaOH.

Electrophysiology. Electrophysiological recording was obtained using the conventional patch-clamp of Hamill et al. (16). The cells were placed in a 0.5-ml-volume bath containing PSS (see *Tissue preparation for microelectrode recording*) and were continuously perfused by gravity at the rate of 1 ml/min. Different test solutions were applied to the cell at 100 μl/min by microcapillaries. Cell membrane currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments). Patch pipettes were pulled from borosilicate glass capillaries and had resistances of 4–7 MΩ. The head-stage ground was connected to an Ag-AgCl pellet that was placed in a side bath filled with the pipette solution and connected to the main bath via an agar bridge containing 3 M KCl. We cancelled the junction potentials between the electrode and the bath by using the voltage pipette offset control of the amplifier. The capacitance of the pipette was cancelled.

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The pipette solution contained (in mM): 125 glutamic acid, 20 KCl, 1 Na_2ATP, 0.01 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 1 EGTA. pH was adjusted to 7.2 using KOH. pCa ~7 was calculated by a computer program developed by Godt and Lindley (15).

Net macroscopic K^+ currents were generated by stepwise 10-mV depolarizing pulses (400 ms duration, 5 s intervals) with a constant holding potential of −80 mV from −80 mV up to +60 mV. Signals were filtered at 1 kHz and digitized at 5 kHz. The steady-state current elicited at chosen E_m was calculated as the average of the current recorded during the latest 50 ms of the pulse. Trials were performed in triplicate and averaged together for the same cell to estimate current amplitudes, which were expressed in picoamperes per picofarad (pA/pF) to eliminate differences of cell membrane area between single vascular myocytes.

The IbTx-sensitive current was defined as the difference between outward current recorded in drug-free bath solution and the current after cell superfusion with 100 nM IbTx, a selective blocker of large conductance KCa channel type (20). The 4-AP-sensitive current was defined as the difference between outward current recorded after cell superfusion with 100 nM IbTx and that after cell superfusion with 100 nM IbTx plus 3 mM 4-AP, a blocker of Kv and a K^+-selective channel that carries a voltage-gated noninactivating K^+ current (K_N) channel types and in pulmonary artery smooth muscle cells (27).

Resting E_m was measured in current clamp mode (current = 0) and just after disruption of the patch membrane. Membrane resistance (R_m) was estimated as the slope of the current-voltage curves between −80 mV and −60 mV where no dynamic currents were activated. The membrane capacitance (C_m) was determine by dividing the integral of the capacitive current by amplitude of 10-mV voltage steps starting from −80 mV.

Voltage clamp protocols were generated, and the data were captured with a computer using a Digidata 1200 interface (Axon Instruments) and pClamp 8 software (Axon Instruments). The analysis was carried out using Clampfit 8 and Origin 6 software (Microcal Software, Northampton, MA).

Chemicals. Stock solutions of 4-AP and IbTx were prepared in distilled water and then diluted in PSS at an appropriate concentration. 4-AP solution was dissolved as stock solution buffered to pH 7.4 with HCl. All chemicals were from Sigma (St. Quentin Fallavier, France).

Statistics. Results are expressed as means ± SE. Statistical analysis was made with the unpaired Student's *t*-test or the Mann-Whitney's test when normality test failed (Anderson-Darling test). For comparison between more than two means we used analysis of variance (ANOVA) followed by Dunnett's test or Mood's median test when normality test failed. The number of experiments (n) refers to the number of cells and N to the number of animals. Differences were considered significant when $P < 0.05$. Statistical analysis was realized using Minitab software (Minitab).

RESULTS

Effect of CO on resting E_m of smooth muscle cells from pressurized resistance PA smooth muscle cells. As shown in Fig. 1*A*, IbTx had no effect on resting E_m of PAC arteries, whereas 4-AP depolarized the membrane. Figure 1*B* shows no significant difference between resting E_m recorded before and after IbTx (n = 19, N = 3), whereas 4-AP (n = 9, N = 3) significantly depolarized the membrane ($P < 0.001$, post hoc unpaired *t*-test).

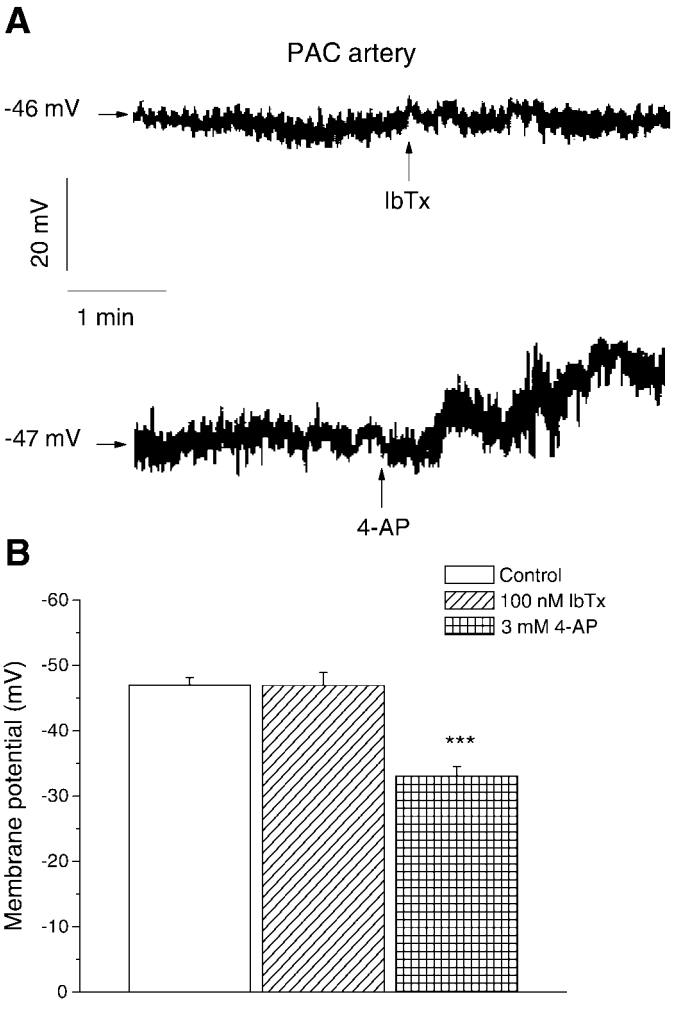

Fig. 1. Effect of iberiotoxin (IbTx) and 4-aminopyridine (4-AP) on resting membrane potential of control (PAC) smooth muscle cells of a pressurized artery. *A*: example of resting membrane potential recording in a pressurized artery before and after action of IbTx (*top*) or 4-AP (*bottom*). *B*: histogram showing mean values of resting membrane potential in control conditions and after action of IbTx or 4-AP. Data are expressed as means ± SE. ***Significant difference compared with control conditions ($P < 0.001$, post hoc unpaired *t*-test).

Resistance PA smooth muscle cells from PACO rats were significantly ($P < 0.001$, post hoc unpaired *t*-test) hyperpolarized (−56.4 ± 2.6 mV, n = 15, Fig. 2*B*) compared with PAC smooth muscle cells (−47.0 ± 1.1 mV, n = 23, N = 3, Fig. 1*B*). Furthermore, as shown in Fig. 2*B*, IbTx (n = 10, N = 3), as well as 4-AP (n = 8, N = 3), significantly depolarized ($P < 0.001$, post hoc unpaired *t*-test) the membrane of a PACO artery. This is clearly shown in Fig. 2*A*. No statistical difference was found between the amplitude of depolarization induced by the two drugs.

Note that the same resting E_m was observed in PAC arteries in the presence of 4-AP and on PACO arteries but in the presence of IbTx or 4-AP ($P > 0.05$, one-way ANOVA and post hoc unpaired *t*-test).

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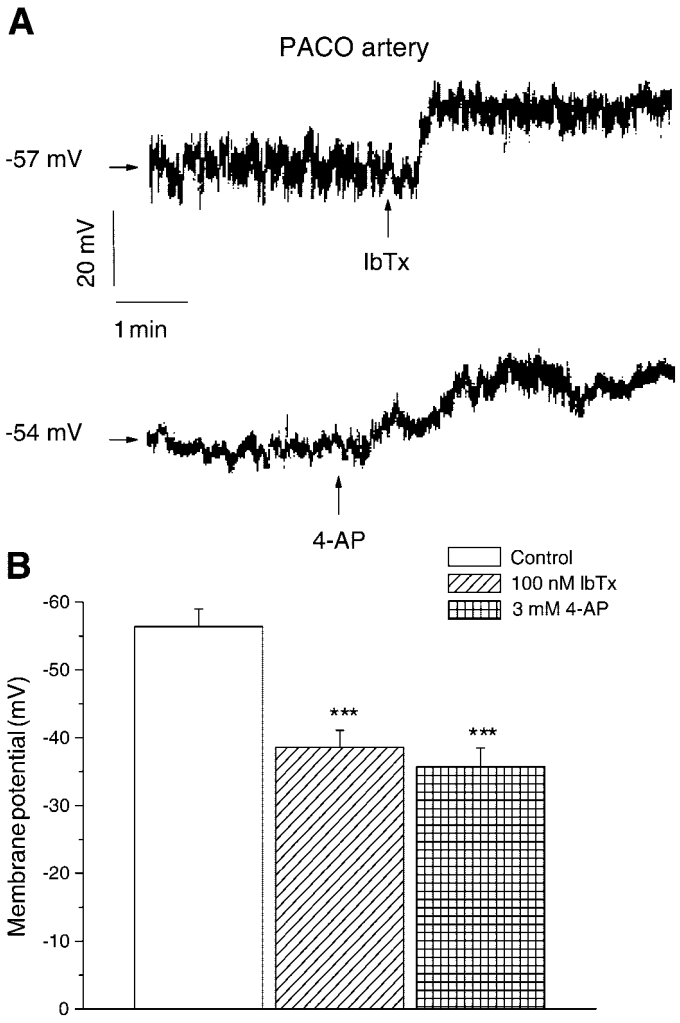

Fig. 2. Effect of IbTx and 4-AP on resting membrane potential of carbon monoxide-exposed (PACO) smooth muscle cells of a pressurized artery. *A*: example of resting membrane potential recording in a pressurized artery before and after action of IbTx (*top*) or 4-AP (*bottom*). *B*: histogram showing mean values (n = 15) of resting membrane potential in control conditions and after action of IbTx or 4-AP. Data are expressed as means ± SE. ***Significant difference compared with control conditions (P < 0.001, post hoc unpaired t-test).

Passive membrane properties of PAC and PACO cells. Using 10-mV voltage steps, we calculated C_m, and no statistically significant difference (P > 0.05, unpaired t-test) was observed between mean values (Fig. 3*A*, *left*) of cells isolated from 12 PAC (n = 18) rats and those of 7 PACO rats (n = 18). Using current-clamp experiments, we measured the resting E_m of PAC cells (n = 18, N = 19), which average −37.8 ± 5.1 mV (Fig. 3*A*, *middle*). This value is in the range of resting E_m already reported in this preparation. Nevertheless, a more hyperpolarized membrane was obtained in PACO cells (−45, 6 ± 4.7 mV, n = 18, N = 7) compared with PAC cells (P < 0.05, unpaired t-test). In the same way, the R_m, which was estimated as the slope of the current-voltage relationship curves between −80 mV and −60 mV, was significantly smaller (P < 0.05, unpaired t-test) in PACO (n = 18, N = 7) cells compared with PAC cells (n = 18, N = 12) (Fig. 3*A*, *right*). As we determined that C_m was not different in cells isolated from the two populations of rats, this was not predictive of the recorded increase of the net outward current density (Fig. 3*B*). The average current density of net outward current at +60 mV in PACO cells represented a 1.7-fold increase compared with PAC cells. Indeed, in PACO cells the average current density was 49.5 ± 8.5 pA/pF (n = 18, N = 7) and was significantly higher than in PAC cells (n = 18, N = 12), in which it was 29.3 ± 3.4 pA/pF (P < 0.05, ANOVA and post hoc unpaired t-test).

Effect of chronic CO on net whole cell current. The effect of chronic CO on whole cell current from a typical experiment is shown in Fig. 4*A*. The cell E_m was held at −80 mV and stepped in 10-mV increments from −80 mV to +60 mV for 400 ms, returning to −80 mV between steps. The outward current was recorded in a 10-pF PAC cell (Fig. 4*A*, *left*) and in a 9.5-pF PACO cell (Fig. 4*A*, *right*). In this example, the cell isolated from the chronic CO-treated rats showed a much larger net outward current. To take into account the cell membrane area, we divided each current amplitude by the respective C_m. Figure 4*B* shows the mean current density-voltage relationship in 18 PAC cells from 12 animals and 18 PACO cells from 7 animals. The current density in PACO cells was markedly and significantly higher (P < 0.05, ANOVA) than in cells isolated from PAC animals (49.5 ± 8.5 pA/pF and 29.3 ± 3.4 pA/pF at +60 mV, respectively). Over the range of potentials where the currents obviously activated (i.e., −40 to −50 mV), chronic CO tended to increase outward current (Fig. 4*B*, *inset*).

Effect of K^+ channel blockers on CO-increased current. The nature of the current activated by chronic CO was further examined with specific K^+ channel blockers. Previous studies have described three major K^+ channels in PA smooth muscle cells, an IbTx-sensitive KCa channel (30) and two 4-AP-sensitive Kv channels (27, 28). Outward currents recorded in a 9.5-pF cell isolated from PAC rat (Fig. 3*A*) exhibited little sensibility to superfusion with 100 nM IbTx, whereas a subsequent addition of 3 mM 4-AP in the superfusion solution containing 100 nM IbTx blocked a larger component of the current. The mean current density-voltage relationship (Fig. 5*B*) confirmed the nonsignificant effect (P > 0.05, ANOVA and Dunnett's post hoc test) of 100 nM IbTx on PAC cells (n = 7, N = 6) for all voltages tested. In comparison, 3 mM 4-AP significantly decreased the current (P < 0.05, ANOVA and Dunnett's post hoc test, n = 6, N = 5). In contrast to PAC cells, a large component of outward current recorded in cells isolated from PACO rat (6.4 pF) was blocked by 100 nM IbTx (Fig. 6*A*). Subsequent addition of 3 mM 4-AP in the superfusion solution containing 100 nM IbTx reduced the residual outward current like in PAC cells. The mean current density-voltage relationship confirmed the large effect of IbTx on PACO cells (Fig. 6*B*).

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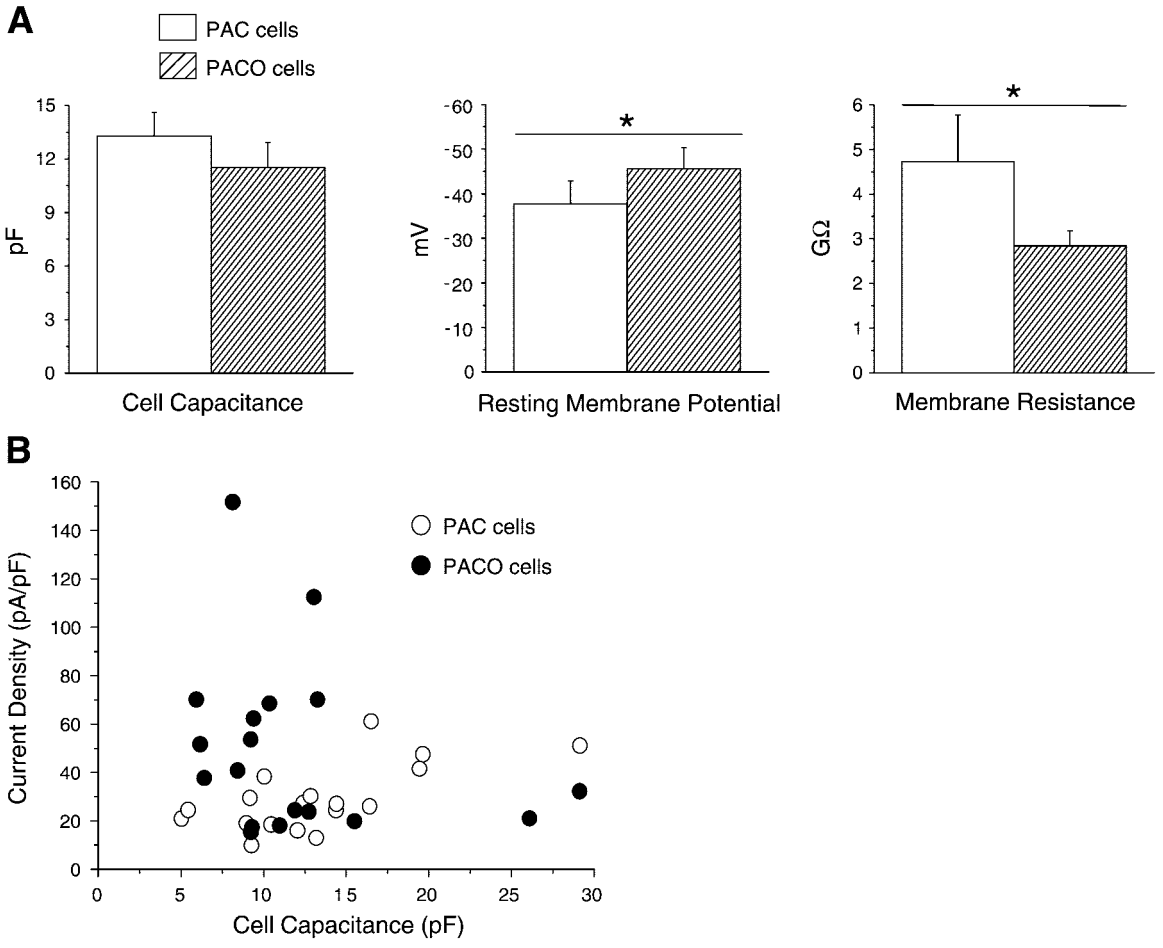

Fig. 3. Passive membrane properties. *A*: comparison of cell capacitance (*left*), resting membrane potential (*middle*), and membrane resistance (*right*) of PAC and PACO cells. Details for determination of these passive membrane properties are given in MATERIALS AND METHODS. Results represent the means ± SE. $*P < 0.05$, significant difference using unpaired *t*-test between each population of isolated cells. *B*: scatter plot showing the membrane density of net current elicited at +60 mV in isolated PAC and PACO cells. Current density is plotted as a function of cell capacitance. Each symbol represents a single cell.

Indeed, in six PACO cells isolated from three animals, the effect of IbTx was significant ($P < 0.05$, ANOVA and Dunnett's post hoc test). Furthermore, over the range of potentials where the currents obviously activated (i.e., −40 to −50 mV), IbTx decreased the outward current, and the resting E_m (estimated to the E_m where the current density was 0 pA/pF) was shifted to more positive values (Fig. 6*B*, *inset*). (Note that this effect was not significant in PAC cells; Fig. 5*B*, *inset*.) 4-AP still significantly decreased ($P < 0.05$, ANOVA and Dunnett's post hoc test) outward current in PACO cells ($n = 6$, $N = 3$).

Effect of chronic CO on IbTx- and 4-AP-sensitive currents. The magnitude of IbTx-sensitive current can be obtained by subtracting the net outward current observed in normal PSS solution from the outward current recorded in the presence of 100 nM IbTx, thereby obtaining the IbTx-sensitive current. In Fig. 7*A*, the current density-voltage relationship for this difference is shown. Compared with PAC cells ($n = 7$, $N = 6$), this IbTx-sensitive current was significantly increased ($P < 0.05$, Mood's median test) in PACO cells ($n = 6$, $N = 3$). At +60 mV, this current represented ~44% (17.5 ± 7.5 pA/pF) of net outward current in PACO cells and ~9% (2.8 ± 0 .5 pA/pF) in PAC cells. We showed that subsequent addition of 4-AP in the bath induced an additional decrease of the outward current in both type of cells. The magnitude of the outward current inhibited by 3 mM 4-AP was obtained by subtracting the outward current observed in the presence of IbTx solution from the outward current recorded in the presence of 100 nM IbTx plus 3 mM 4-AP. The current density-voltage relationship for this 4-AP-sensitive current is shown in Fig. 7*B*, and we observed no significant difference ($P > 0.05$, Mood's median test) between PAC and PACO cells. For example, the 4-AP-sensitive current at +60 mV represented ~55% of net current (13.7 ± 2.2 pA/pF) in PAC cells ($n = 6$, $N = 5$). This is not different from the 4-AP-sensitive current observed in PACO cells (14.4 ± 3.6 pA/pF, $n = 6$, $N = 3$) but represented ~38% of net current. Furthermore, although the 4-AP- and IbTx-sensitive currents are significantly different in PAC, these currents are not different in PACO cells.

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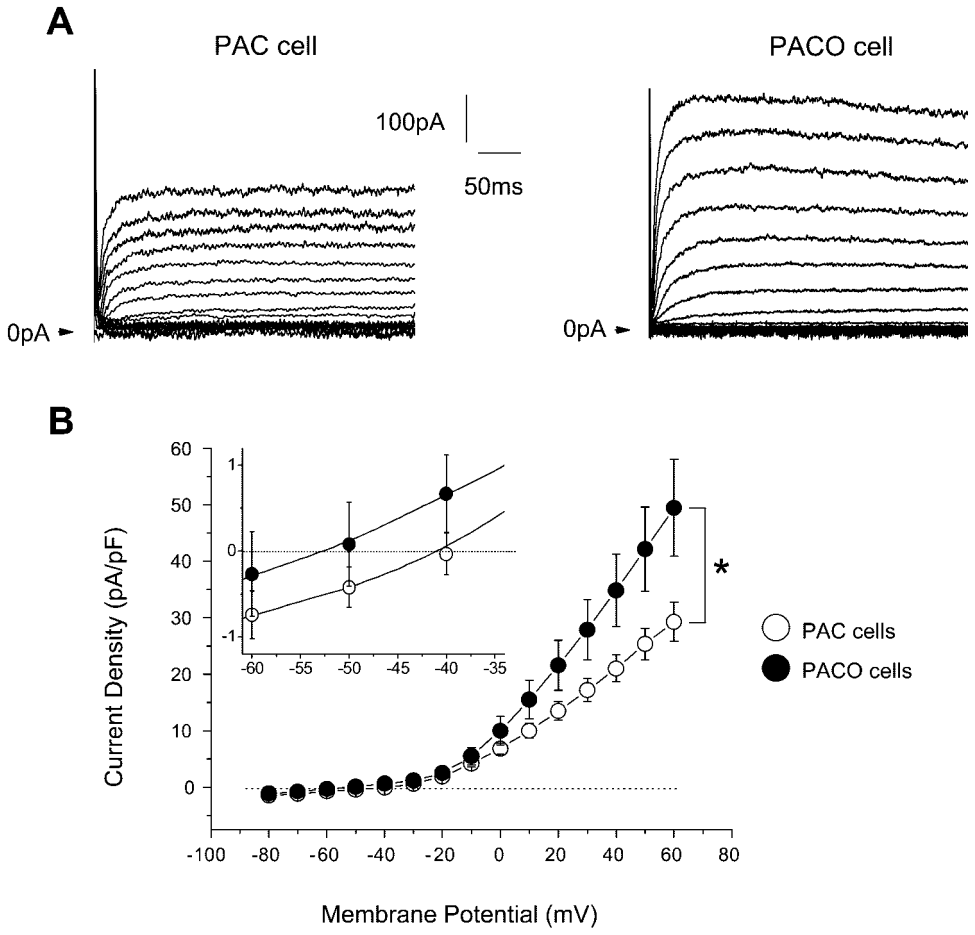

Fig. 4. Net whole cell current and current density-voltage relationships during voltage steps in isolated PACO and PAC cells. *A*: family of currents elicited by incremental 10-mV depolarizing steps from −80 to +60 mV in cells isolated from PAC and PACO rats. *B*: current density-voltage relationship showing an increase of net outward current amplitude in cells dissociated from PACO rat. *Inset*: an expanded scale of the same current density-voltage relationship between −60 and −35 mV. Results represent the means ± SE. $*P < 0.05$, significant difference using unpaired *t*-test between each population. After a 1-way ANOVA, between-population comparison was made between current density obtained in PAC and PACO isolated cells within each membrane potential.

DISCUSSION

This study demonstrates that an IbTx-sensitive K^+ current was activated in resistance PA smooth muscle cells of rats exposed for 3 wk to CO. This could explain the hyperpolarization and the modification of E_m reactivity to K^+ channel blockers in resistance PA cells from PACO rats.

Pharmacological relevance of 530-ppm chronic CO inhalation. CO is an endogenously generated gas as well as an ubiquitous environmental gas. The two major sources of exogenous CO exposure are automotive emissions and cigarette smoke. Indeed, cigarette smoke, a frequently cited source of indoor CO, contains CO levels ranging from 35 to 1,000 ppm (38). In addition, exposure to high levels of CO can occur in numerous occupational settings, such as those experienced by tunnel workers (9). Not surprisingly then, hospital admissions, mortality, and morbidity are correlated with CO levels in air (19, 24). Effects of exogenous CO result primarily from its competition with oxygen binding to hemoglobin; nevertheless, a direct action of CO has also been demonstrated on vascular smooth muscles (39).

Chronic CO-induced modifications of passive membrane properties of resistance PA cells. Results from the present investigation demonstrate that chronic CO hyperpolarized the membrane and caused an increase of IbTx-sensitive outward current without changing the C_m of the cells. Because IbTx is a selective pharmacological blocker of the large conductance KCa channel type (20), we suppose that CO increased large conductance KCa current. Furthermore, the average cell R_m decreased, as would be excepted if CO activated outward current. If the hyperpolarization of the membrane resulted from the enhancement of IbTx-sensitive current, then IbTx should be a potent depolarizing agent in PACO cells. Indeed, we found that IbTx depolarized membranes of PACO cells but not those of PAC cells (estimated to the E_m where the current density was 0 pA/pF); this was corroborated by records of resting E_m on pressurized resistance PA cells. PA smooth muscle cells have a high resting membrane input resistance, which we found to be 4.7 GΩ; this is close to the value obtained by Park et al. (29). This corresponds to very low channel activity near the resting E_m (and with 100 nM intracellular Ca^{2+}) and has the consequence that opening of very few channels can cause a substantial hyperpolarization (25). The mean R_m was decreased from 4.7 to 2.8 GΩ under chronic CO. This decrease in resistance corresponded to an increase of conductance of ~530 pS (1/1.89 GΩ).

KCa current is increased in PACO cells. Acute exogenous CO has been shown to activate KCa and Kv channels, respectively, in rat tail artery smooth muscle

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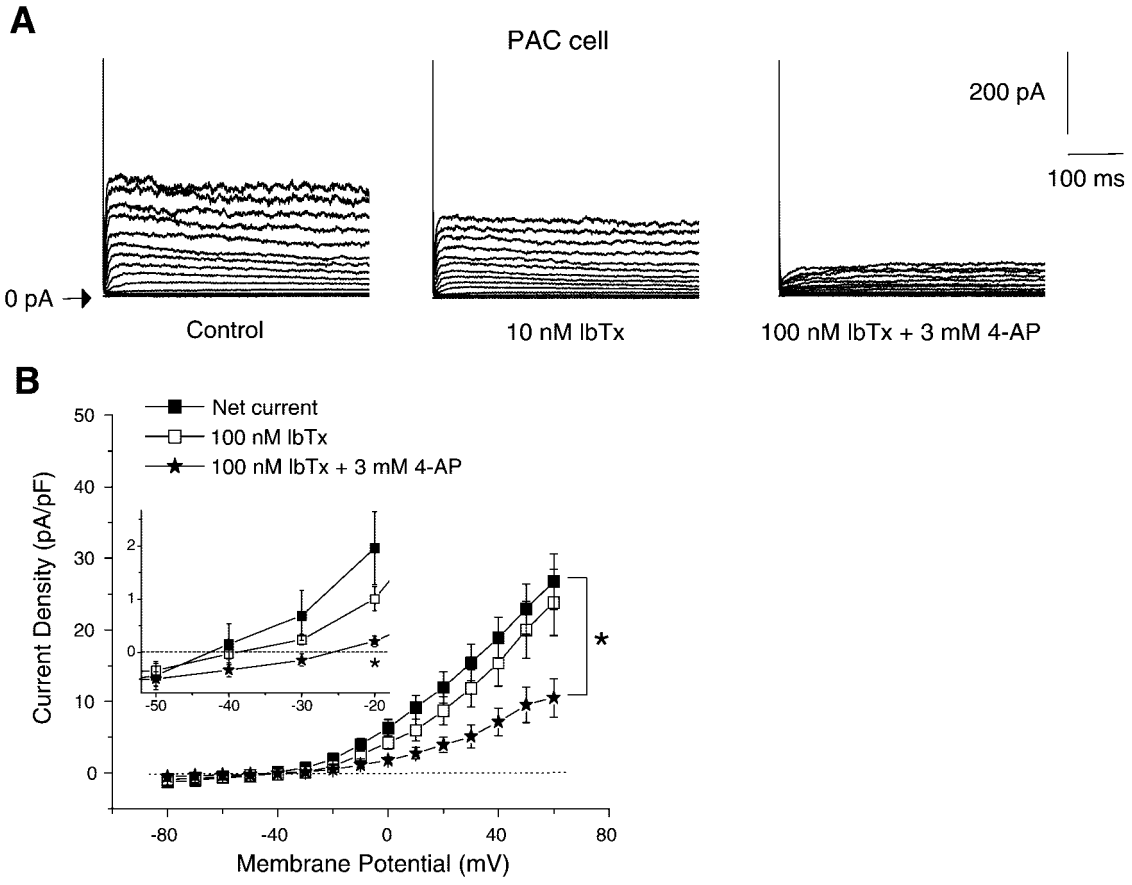

Fig. 5. Effect of IbTx and 4-AP on whole cell current and current density-voltage relationships during voltage steps in isolated PAC cells. *A*: IbTx (100 nM) blocked a small component of outward current; in contrast, 4-AP (3 mM) blocked a large IbTx-insensitive component of current. *B*: current-voltage relationships showing the effect of IbTx and 4-AP on isolated PAC cells. *Inset*: an expanded scale of the same current density-voltage relationship between −50 and −20 mV. Results represent the means ± SE. $*P < 0.05$, significant difference using unpaired *t*-test between each condition (net current, with IbTx, with IbTx and 4-AP). After a 2-way ANOVA between these conditions, comparison was made between current density obtained in each conditions and within each membrane potential.

cells (40) and in human jejunal circular smooth muscle cells (14). An effect of CO on the delayed rectifier K^+ channel, already observed in visceral smooth muscle cells, and on the K_N current was excluded, because the increase of outward current we observed was not sensitive to 4-AP, a general blocker of Kv channels. After chronic CO exposure we saw an increase of the global K^+ current, and this was associated with an increase of IbTx-sensitive current, probably large conductance KCa current. Thus these observations suggest that this increase of net outward current by exposure to CO is largely mediated by KCa channels. This is supported by experiments showing that an inhibitor of HO (leading probably to a decrease of endogenous CO) reduced KCa currents (43).

Our results demonstrate that Kv and KCa currents coexist in both populations of cells (PAC and PACO) but that their relative contributions in global current changed. Before CO treatment, the large conductance KCa current component accounts for ~9% and tended to increase to ~44% after chronic CO treatment. However, the Kv current component decreased from ~55 to ~38% after CO exposure. These results suggest that CO treatment could induce a differential expression of K^+ channels in resistance PA cells. In this regard a recent study has already demonstrated a differential expression of Kv and KCa channels in vascular smooth muscle cells after 1-day culture (35).

Because in our experiments we compared electrical properties of the cells using conventional whole cell patch-clamp techniques under the same experimental conditions and, in particular, using the same intracellular free [Ca^{2+}], we cannot explain this increase of KCa current by an increase of intracellular Ca^{2+}. Furthermore, this increase of outward current persisted after several minutes of equilibration of intrapipette solution in the cells. We cannot exclude an effect of CO on KCa channels via activation of guanosine 3′,5′-cyclic monophosphate (cGMP)-dependent protein kinase (2, 39). Indeed, expression of this kinase or another part of the cGMP pathway could be altered. A modification of the Ca^{2+} dependence of KCa channels, as well as a direct chemical modification of KCa channels by CO, is not excluded,

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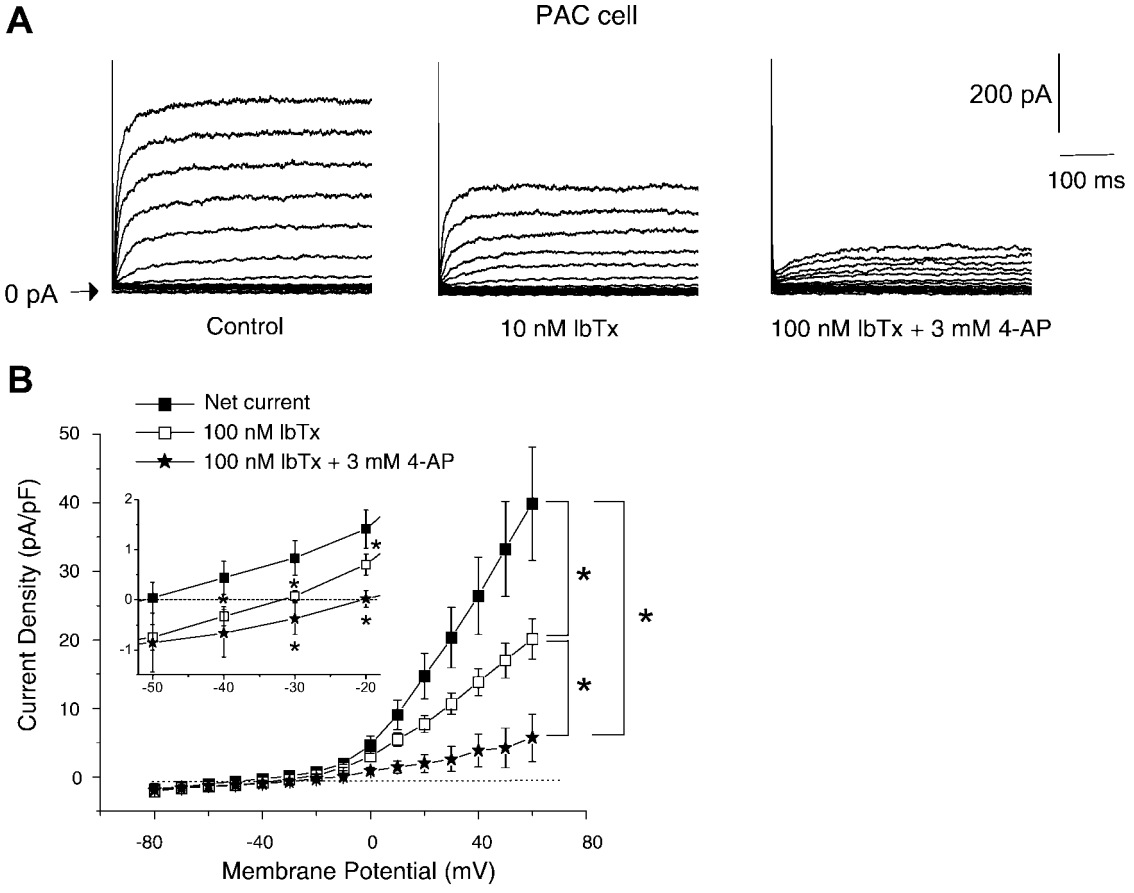

Fig. 6. Effect of IbTx and 4-AP on whole cell current and current density-voltage relationships during voltage steps in isolated PACO cells. *A*: IbTx (100 nM) blocked a large component of outward current in the PACO cells, and 4-AP (3 mM) blocked almost the same component of IbTx-insensitive current in both populations of cells. *B*: current-voltage relationships showing the effect of IbTx and 4-AP on isolated PACO cells. *Inset*: an expanded scale of the same current density-voltage relationship between −50 and −20 mV. Results represent the means ± SE. $*P < 0.05$, significant difference using unpaired *t*-test between each condition (net current, with IbTx, with IbTx and 4-AP). After a 2-way ANOVA between these conditions, comparison was made between current density obtained in each condition and within each membrane potential.

as this was observed in tail artery smooth muscle cells (39). An increase of spark activity could also explain this increase of KCa current, but we should have observed spontaneous transient outward current (STOCs) as we had already observed in these cells (37). Furthermore, this should be tested using perforated-patch experiments on less-modified Ca^{2+} homeostasis by intrapipette solution. More experi-

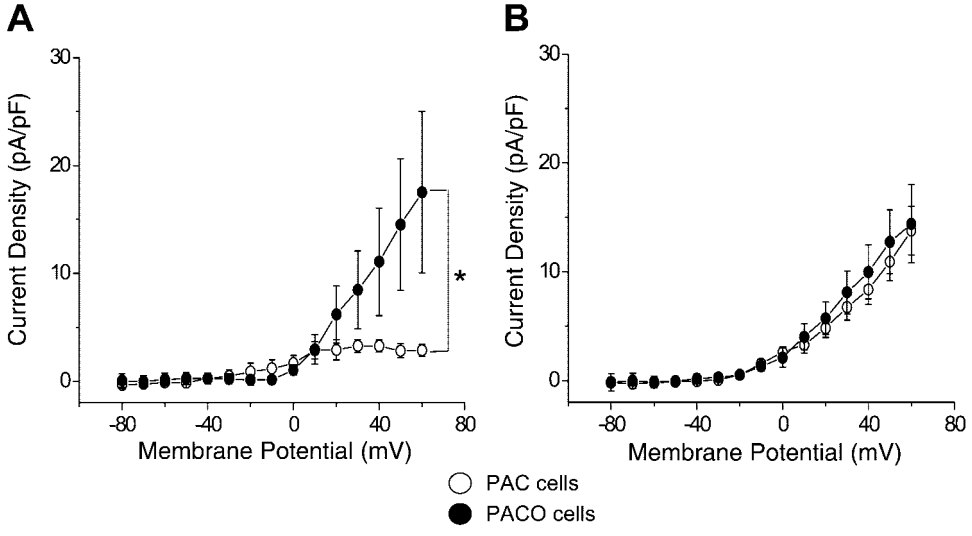

Fig. 7. IbTx- and 4-AP-sensitive current density during voltage steps in isolated PACO and PAC cells. Current density-voltage relationship showing that the component of IbTx-sensitive current was significantly greater in PACO cells than in PAC cells at the indicated membrane potential, an increase of net outward current amplitude in cells dissociated from PACO rats. The 4-AP-sensitive current was no different between these populations of cells. Results represent the means ± SE. $*P < 0.05$, significant difference using unpaired *t*-test between each population. After a 1-way ANOVA, between-population comparison was made between current density obtained in PAC and PACO isolated cells within each membrane potential.

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ments are needed to explore how CO increases KCa current and how exposure for 3 wk to CO can induce an increase of KCa channel expression leading to an increase in the number of functional channels.

Functional impact of KCa channels after chronic CO exposure. CO is also an endogenously generated gas that regulates vascular tone (39). The predominant biological source of CO is from degradation of heme by HO (36). At least two isoforms of HO, HO-1 and HO-2, an inducible and a constitutive isoform, respectively, have been identified and are present in vascular smooth muscle cells (12). Most experiments suggest that endogenously generated CO affects vascular tone in physiological and pathological conditions, but these experiments were performed with an activator/blocker of HO to activate or inhibit the endogenous CO production (39). Moreover, there is no direct evidence that these effects were due to CO, because the effect of chronic exogenous CO on vascular tone is lacking. Furthermore, the physiological and chronic importance of endogenously generated CO on K^+ channel functions was not directly studied. Indeed, only studies using a blocker of HO to inhibit the endogenous CO production and then the changes in K^+ channel function were performed (18, 21, 43). Interestingly, Zhang et al. (43) demonstrated that an IbTx-sensitive current is decreased after inhibition of HO, suggesting an activating effect of endogenous CO on vascular KCa currents.

PA smooth muscle cells have a high density of KCa channels, and these channels (through STOCs) regulate resting E_m of PA smooth muscle cells (3, 37). It has been suggested that, in PA smooth muscle cells, the major role of KCa channels is a negative-feedback mechanism that acts to counteract membrane depolarization (42) and thus membrane contraction. Indeed, the high R_m of PA smooth muscle cells, coupled with the large unitary conductance of large conductance KCa channels, provides this KCa channel with a highly favorable environment in which to buffer membrane depolarization (5). Because they are activated by membrane depolarization and an increase of intracellular Ca^{2+}, they play a minor role in regulating baseline tone of PA. Indeed, although charybdotoxin has little effect under this conditions, this blocker enhances the increase of PA pressure to hypoxia (26).

We found significant hyperpolarization of pressurized resistance PA smooth muscle cells after CO, and we demonstrated that resting E_m of PACO smooth muscle cells, and not of PAC smooth muscle cells, was sensitive to IbTx. This suggests that KCa were activated after chronic CO exposure and could explain the hyperpolarization of PACO arteries. Indeed, we showed that KCa current was increased in PACO smooth muscle cells. This membrane hyperpolarization not only inactivates voltage-dependent Ca^{2+} channels but also inhibits agonist-induced increase of inositol triphosphate and reduces Ca^{2+} sensitivity, leading to vasorelaxation (39). In some cardiovascular pathologies such as systemic arterial hypertension, the expression and activity of KCa are increased, leading to nullified increased tendency of arteries to constrict (22).

In contrast to chronic CO, chronic hypoxia-induced pulmonary hypertension is associated with decreased KCa channel activity (30), leading to more depolarized and thus more excitable smooth muscle cells. Furthermore, HO-1 is transiently increased by chronic hypoxia (7) whenever sustained activation prevents the development of hypoxic pulmonary hypertension (8). Furthermore, this increase of KCa currents induced by chronic CO (exogenously or endogenously) may provide a novel target for therapy during pulmonary hypertension as nitric oxide does (1).

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