Effect of chronic hypoxia on agonist-induced tone and calcium signaling in rat pulmonary artery

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IN MANY MAMMALIAN SPECIES including human, a prolonged decrease in alveolar oxygen tension induces a selective pulmonary vasoconstriction that is generally accompanied by an important vascular remodeling and a sustained pulmonary arterial hypertension (PAHT) (34, 35, 41). Chronic hypoxia (CH)-induced PAHT results from a combination of polycythemia and structural and functional changes in the pulmonary vascular bed (1, 17, 35). Structural changes involve cellular hypertrophy and hyperplasia and deposition of additional matrix that decreases the lumen of the vessels and reduces the elasticity of the arterial wall (17, 24, 46). These structural changes also contribute to the functional modification of pulmonary arterial reactivity. Despite their pathophysiological importance, the molecular and cellular mechanisms underlying these phenomena are not fully elucidated.

Under normoxic conditions, pulmonary arterial tone is controlled by both membrane potential (8, 49) and a variety of circulating and locally released mediators such as endothelin (ET)-1, angiotensin II (ANG II), serotonin, and ATP (4, 7, 25). Several studies have investigated the effect of CH on membrane potential or ionic currents and reactivity to vasoconstrictors. In both animals and humans, CH depolarizes pulmonary vascular smooth muscle cells as a consequence of a dysfunction of voltage-gated K+ channels, and this depolarization secondarily increases the resting intracellular Ca2+ concentration ([Ca2+]i) (31, 32, 43, 45, 48). It has also been demonstrated that CH in rats increases ET-1 gene expression and plasma ET-1 levels as in PAHT in humans (1, 9, 10, 23). CH also increases ANG II-converting enzyme activity in rats (29, 30, 50), suggesting that both mediators are implicated in PAHT. Although ET-1 and ANG II inhibit voltage-gated K+ channels under normoxic conditions, their effect is differentially altered by CH (33, 37, 43). Regarding the effect of CH on the reactivity to agonists, considerable variability is evident in the literature depending on the animal species, the duration and degree of exposure to CH, and the considered portion of the pulmonary vascular bed (6, 22, 26–28). Moreover, although an increase in [Ca2+]i is recognized as the key step in the activation-contraction process and thus in the vascular reactivity, to the best of our knowledge, little information is available about the effect of CH on agonist-induced Ca2+ signaling in pulmonary vascular smooth muscle. Very recently, Shimoda et al. (42) have shown an inhibitory effect of CH on the ET-1-induced transient [Ca2+]i increase in intrapulmonary arteries. Under normoxic conditions, our laboratory has previously shown (13, 15, 20, 40) that ET-1 as well as ANG...
II and ATP induces a complex [Ca\(^{2+}\)]\(_i\) response in myocytes isolated from the rat main pulmonary artery (MPA). This response is composed of a series of [Ca\(^{2+}\)]\(_i\) oscillations, which are mainly due to a cyclic release of stored Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) via an inositol 1,4,5-trisphosphate [Ins\((1,4,5)P_3\)]-sensitive pathway (13, 15, 20, 40). On one hand, these Ca\(^{2+}\) oscillations directly account for the main part of the contractile response, and on the other hand, they trigger an oscillatory Ca\(^{2+}\)-activated Cl\(^-\) current (14, 20). This current depolarizes pulmonary arterial myocytes to the threshold activation of the voltage-dependent Ca\(^{2+}\) channel (3, 14, 20), thus inducing a Ca\(^{2+}\) influx responsible for an additional component of the agonist-induced contraction.

A better knowledge of the cellular effect of agonists under CH would be of interest to further understand the pathophysiology of the pulmonary circulation. The current study was thus designed to investigate, in the rat MPA, the effect of CH on both the agonist-induced contraction and the [Ca\(^{2+}\)]\(_i\) response. Tissues were obtained from rats maintained either in a hypobaric chamber (50.5 kPa) for 14 days (chronically hypoxic rats) or under normoxic conditions (control rats). Indo 1 microspectrofluorometry was used in freshly isolated myocytes to measure [Ca\(^{2+}\)]\(_i\), and isometric contraction was measured in arterial rings. We have observed that the CH-induced decrease in the reactivity to ET-1 and ANG II was due to both an effect on Ca\(^{2+}\) signaling and a decrease in the Ca\(^{2+}\) sensitivity of the contractile apparatus. The main effect of CH on Ca\(^{2+}\) signaling, i.e., the loss of agonist-induced Ca\(^{2+}\) oscillations, appears to depend on an action at the site of Ca\(^{2+}\) uptake mechanisms.

**MATERIALS AND METHODS**

**CH.** Adult male Wistar rats (aged 8–10 wk, weighing 220–240 g) were separated into two groups. One group (control or normoxic rats) was housed in room air at a normal atmospheric pressure (101 kPa). The other group (hypoxic rats) was maintained in a hypobaric chamber for 14 days. The pressure in the chamber was reduced to 0.5 atmosphere (50.5 kPa) with an electrically driven pump. The chamber was opened for 15–30 min twice a week. Pulmonary hypertension was assessed by measuring the ratio of right ventricle (RV) to left ventricle plus septum (LV+S) weight (5, 27, 43).

**Tissue preparation.** At completion of the exposure, the rats were anesthetized with an intraperitoneal injection of 40 mg of ethyl carbamate. The heart and lungs were removed en bloc. The MPA was then dissected under binocular control, and the adventitial and intimal layers were removed. For contraction experiments, rings (3 mm in length) were prepared. For cell dissociation, the MPA was cut into several pieces (1 × 1 mm), incubated for 10 min in low-Ca\(^{2+}\) (200 μM) physiological saline solution (PSS; composition given in *Solutions and application of agonists*), and then incubated in low-Ca\(^{2+}\) PSS containing 0.5 mg/ml of collagenase, 0.4 mg/ml of Pronase, 0.06 mg/ml of elastase, and 3 mg/ml of bovine serum albumin at 37°C for two successive periods of 20 min each, with fresh enzymes each time. After this sequence, the solution was removed, and the arterial pieces were incubated again in a fresh enzyme-free solution and triturated with a fire-polished Pasteur pipette to release the cells. The cells were stored on glass coverslips at 4°C in PSS containing 0.8 mM Ca\(^{2+}\) and used on the same day.

**Isometric contraction measurement.** Isometric contraction was measured in rings from the MPA that were mounted between two stainless steel clips in vertical 20-ml organ baths of a computerized isolated organ bath system (IOX, EMKA Technologies, Paris, France). The baths were filled with Krebs-Henseleit solution (composition in mM: 118.4 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 25 NaHCO\(_3\), and 11.1 D-glucose, pH 7.4) maintained at 37°C and bubbled with a 95% O\(_2\)/5% CO\(_2\) gas mixture. The upper stainless clip was connected to an isometric force transducer (EMKA Technologies). As determined in preliminary experiments, tissues were set at optimal length by equilibration against a passive load of 10 and 20 mN for rings obtained from normoxic and hypoxic rats, respectively. At the beginning of each experiment, a K\(^+\)-rich (80 mM) solution obtained by substituting an equimolar amount of KCl for NaCl in the Krebs-Henseleit solution was repeatedly applied to obtain at least two contractions similar in both amplitude and kinetics. This contraction served as a reference response that was used to normalize subsequent contractile responses. A cumulative concentration-response curve to ANG II (0.1 nM to 1 μM) or ET-1 (0.1–100 nM) was then constructed. A concentration increment was made once the maximal contractile effect of the preceding concentration had been recorded. For ET-1, the maximal force was obtained after a 15- to 18-min application of ET-1, and the incremental concentration of ET-1 was added after such a duration in our experiments, whereas for ANG II, the incremental concentration was added at the top of the response to the preceding concentration obtained for a 4- to 5-min application because the ANG II-induced response is transient. Successful removal of the endothelium was confirmed by the inability of acetylcholine (1 μM) to induce >10% of relaxation in phenylephrine (1 μM)-contracted rings.

**[Ca\(^{2+}\)]\(_i\) measurements.** To assess the dynamic changes in [Ca\(^{2+}\)]\(_i\) of individual arterial myocytes, we used the [Ca\(^{2+}\)]\(_i\)-sensitive fluorophore indo 1. The cells were loaded with indo 1 by incubation in PSS containing 1 μM indo 1-AM for 25 min at room temperature and then washed in PSS for 25 min. The coverslip with the attached cells was then mounted in a perfusion chamber. The recording system included a Nikon Diaphot inverted microscope fitted with epifluorescence (Nikon, Tokyo, Japan). A single cell among those on the coverslip was tested through a window slightly larger than the cell. The studied cell was illuminated at 360 nm and counted simultaneously at 405 and 480 nm by two photomultipliers (P100, Nikon). The 405- to 480-nm fluorescence ratio was calculated on-line and displayed with the two voltage signals on a monitor. [Ca\(^{2+}\)]\(_i\), was estimated from the fluorescence ratio (12), with a calibration for indo 1 determined within the experiments (14).

**Solutions and application of agonists.** The external PSS contained (in mM) 130 NaCl, 5.6 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 11.1 D-glucose, and 10 HEPES, pH 7.4 with NaOH. Ca\(^{2+}\)-free PSS was prepared by replacing CaCl\(_2\) with 0.4 mM EGTA. ET-1, ANG II, ATP, and caffeine were applied to the recorded cell by pressure ejection from a glass pipette located close to the cell for the period indicated on the records. It was verified in control experiments that no change in [Ca\(^{2+}\)]\(_i\), was observed during test ejections of PSS. Generally, each record of [Ca\(^{2+}\)]\(_i\), response to the different agonists was obtained from a different cell. Each type of experiment was repeated for the number of cells indicated in the text. Experiments were done at room temperature (20–22°C).
**Chemicals and drugs.** Collagenase (type CLS1) was from Worthington Biochemical (Freehold, NJ), Pronase (type E), elastase (type 3), bovine serum albumin, ANG II, ATP, cyclopiazonic acid (CPA), ET-1, methoxyverapamil (D-600), and thapsigargin were from Sigma (Saint Quentin Fallavier, France). Caffeine was from Merck (Darmstadt, Germany). Indo 1 was from Calbiochem (France Biochem, Meudon, France). CPA, D-600, indo 1, and thapsigargin were dissolved in DMSO. The maximal concentration of DMSO used in our experiments was <0.1% and had no effect on the mechanical activity of rings or the resting value of or the variation in [Ca$^{2+}$]$_i$, induced by agonists in the cells.

**Analysis of data.** [Ca$^{2+}$]$_i$ responses to ANG II, ET-1, and ATP were analyzed by comparing the resting [Ca$^{2+}$]$_i$ value, the relative amplitude of the first peak of the response, the percentage of responding cells, the percentage of cells generating [Ca$^{2+}$]$_i$ oscillations (oscillatory cells), and the number of Ca$^{2+}$ oscillations in cells obtained from both control and CH rats. The amount of cytosolic mobilized Ca$^{2+}$ was estimated by determining the area under the [Ca$^{2+}$]$_i$ curve. The falling part of the transient caffeine-induced [Ca$^{2+}$]$_i$ response was kinetically analyzed. It was composed of two successive linear and exponential phases that were described by the following equations as a function of time ($t$): [Ca$^{2+}$]$_i$ = $A$t + $b$ and [Ca$^{2+}$]$_i$ = [Ca$^{2+}$]$_{i0}$ - $B$e$^{-}\tau t$ for phases 1 and 2, respectively, where $A$ is the slope coefficient of phase 1, $b$ is the peak [Ca$^{2+}$]$_i$ value in phase 1, $\tau$ is the time constant of phase 2, [Ca$^{2+}$]$_{i0}$ is the [Ca$^{2+}$]$_i$ value at the end of phase 2, and $B$ is the [Ca$^{2+}$]$_i$ value at the onset of phase 2. $A$ and $\tau$ were compared between cells obtained in control and CH rats, respectively. In contraction experiments, the concentration of ANG II or ET-1 inducing 50% of the maximal response (EC$_{50}$) was graphically determined from the mean cumulative concentration-response curve.

Results are expressed as means ± SE, with $n$ the number of myocytes in the sample. Significance was tested by means of Student’s t-test at a $P$ value of <0.05. Qualitative data (frequency of responding cells) were tested by means of $\chi^2$-test.

## RESULTS

**Effect of CH on RV weight.** In rats exposed to hypobaric hypoxia for 14 days, RV-to-LV+S ratio values significantly increased compared with those in control rats (0.46 ± 0.2, $n$ = 10, and 0.30 ± 0.01, $n$ = 18, respectively; $P$ < 0.05). This RV hypertrophy was the consequence of the development of PAHT.

| Table 1. Effect of CH on resting and ET-1, ANG II, and ATP-induced [Ca$^{2+}$]$_i$ responses in MPA myocytes in control and CH rats |
|----------------|------------------|------------------|------------------|------------------|
|                | Resting [Ca$^{2+}$]$_i$, nM | Peak [Ca$^{2+}$]$_i$, nM | Responding Cells, % | Oscillatory Cells, % | No. of Oscillations |
| ET-1 (100 nM)  |                      |                  |                    |                    |                   |
| Control        | 86                  | 69 ± 11          | 586 ± 105          | 85                 | 84                | 6 ± 1.7           |
| CH             | 85                  | 109 ± 16*        | 480 ± 117          | 65*                | 15*               | 3.6 ± 1*          |
| ANG II (10 µM) |                      |                  |                    |                    |                   |
| Control        | 20                  | 60 ± 17          | 474 ± 68           | 90                 | 82                | 5.2 ± 1.2         |
| CH             | 20                  | 99 ± 11*         | 356 ± 50           | 40*                | 33*               | 2.6 ± 0.6*        |
| ATP (100 µM)   |                      |                  |                    |                    |                   |
| Control        | 21                  | 62 ± 8           | 491 ± 85           | 90                 | 87                | 4.5 ± 1.6         |
| CH             | 15                  | 99 ± 8*          | 338 ± 41           | 35*                | 27*               | 3.1 ± 1.8*        |

Values are means ± SE; $n$, no. of myocytes in a sample. CH, chronic hypoxia; ET-1, endothelin-1; ANG II, angiotensin II; [Ca$^{2+}$]$_i$, intracellular Ca$^{2+}$ concentration; MPA, main pulmonary artery. *$P$ < 0.05 between normoxic (control) and CH rats.
The two phases were slowed down in cells from CH rats. The slope coefficient \( (A) \) of phase 1 and the time constant \( (\tau) \) of phase 2 were significantly decreased and increased, respectively (Table 2).

**Effect of CPA on the caffeine-induced \([\text{Ca}^{2+}]_i\) response.** In this set of experiments, the effect of CPA (5 and 10 \( \mu \)M), another specific inhibitor of the SR \( \text{Ca}^{2+} \) pump in the pulmonary artery (11), on the caffeine-induced \([\text{Ca}^{2+}]_i\) response in MPA myocytes from control rats was examined. CPA significantly increased the resting \([\text{Ca}^{2+}]_i\) value from 69 ± 11 \( (n = 86) \) to 118 ± 35 \( (n = 25) \) and 125 ± 43 \( (n = 15) \) nM in the presence of 5 and 10 \( \mu \)M CPA, respectively. As in myocytes from CH rats, the two phases of the falling part of caffeine-induced \([\text{Ca}^{2+}]_i\) response were significantly delayed by CPA in myocytes from control rats (Fig. 5, Table 2). Finally, CPA had no effect on the falling part of the caffeine-induced \([\text{Ca}^{2+}]_i\) response in myocytes from CH rats.

**Effect of CH on ANG II- and ET-1-induced contractile responses.** In MPA rings from control rats, ET-1 (0.1–100 nM) and ANG II (0.1–1 \( \mu \)M) induced concentration-dependent contractions. The maximal ET-1- and ANG II-induced responses were 140.1 ± 12.6 \( (n = 6) \) and 72.63 ± 9.8% \( (n = 4) \), respectively, of the 80 mM KCl-induced contraction (Fig. 6). The mean EC\(_{50}\) values were 1.9 and 0.6 nM for ET-1 and ANG II, respectively. In MPA rings from CH rats, the maximal forces induced by ET-1 and ANG II were reduced by 30 and 30.2%, respectively (Fig. 6, A and B, respectively). CH also significantly increased the mean ANG II EC\(_{50}\) to
We did verify that the raw amplitude of the 80 mM KCl-induced contraction was not different in MPA rings from control and CH rats (2,490.58 ± 403 mg, n = 13, and 2,335.53 ± 579.3 mg, n = 16, respectively; P > 0.05).

Finally, we investigated the contractile effect of ET-1 (100 nM) 30 min after pretreatment of the rings with a combination of D-600 (10 μM) and thapsigargin (1 μM). The remaining agonist-induced contraction observed under such experimental conditions reflects the sensitization of the contractile apparatus in vascular smooth muscle (38). After such pretreatment, ET-1-induced contraction was 22 ± 6.6 and 13 ± 4.7% (n = 4; P < 0.05) of the ET-1-induced contraction in the absence of pretreatment in rings from control and CH rats, respectively (Fig. 6C).

**DISCUSSION**

The present study shows that in the rat MPA, CH alters both smooth muscle reactivity and Ca²⁺ signaling in response to a variety of agonists. In vascular tissues obtained from rats exposed for 14 days to CH, we observed a decrease in the reactivity to ET-1 and ANG II that can be ascribed to a complex combined effect on 1) the percentage of cells responding to agonists acting at plasmalemma membrane receptors, 2) Ca²⁺ signaling, and 3) Ca²⁺ sensitivity of the contractile apparatus. The Ca²⁺ reuptake mechanism appears as a CH-sensitive phenomenon that may account for the main effect of CH on Ca²⁺ signaling, i.e., the loss of agonist-induced Ca²⁺ oscillations.

A significant decrease in the percentage of responding cells was observed regardless of the agonist used in the present study. Therefore, this effect was not restricted to a specific type of membrane receptor. Biochemical modulation of the agonist receptor binding step with either a decrease in the number of receptors expressed at the surface membrane or a decrease in the agonist binding affinity may account for this effect. In the absence of binding experiments, this hypothesis cannot be ruled out. However, again, it should be kept in mind that such phenomena could occur in a similar way for different receptors. Alternatively, we would favor the hypothesis of a decrease in the receptor-
respectively, where nary arteries (42, 47, 48) and, more specifically, a
(Table 1) as previously shown by others in intrapulmo-
value at the end of phase 2.

\[ \text{[Ca}^{2+}\text{]i} \]
described by the following equations as a function of time (\( t \)):

\[
\text{[Ca}^{2+}\text{]i} = \text{A}(t) + b \quad \text{[Ca}^{2+}\text{]i}_0 + Be^{-\gamma t}
\]

for phases 1 and 2, respectively, where \( A \) is the slope coefficient of phase 1, \( B \) is the peak \([\text{Ca}^{2+}\text{]i}\), value for phase 1, \( \gamma \) is the \([\text{Ca}^{2+}\text{]i}\), value at the onset of phase 2, \( \tau \) is the time constant of phase 2, and \([\text{Ca}^{2+}\text{]i}_0\) is the \([\text{Ca}^{2+}\text{]i}\), value at the end of phase 2. \( *P < 0.05 \) compared with control.

**Table 2. Effect of CH and CPA on kinetics of caffeine-induced \([\text{Ca}^{2+}\text{]i}\), response in MPA myocytes**

<table>
<thead>
<tr>
<th></th>
<th>( n )</th>
<th>( \text{Slope Coefficient, nM/s} )</th>
<th>( \text{Time Constant, s} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>(-12.8 \pm 2)</td>
<td>(14.8 \pm 4.3)</td>
</tr>
<tr>
<td>CH</td>
<td>16</td>
<td>(-8.1 \pm 2.1^*)</td>
<td>(23.1 \pm 5.3^*)</td>
</tr>
<tr>
<td>CPA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 ( \mu \text{M} )</td>
<td>25</td>
<td>(-8.3 \pm 1.7^*)</td>
<td>(28.8 \pm 6.6^*)</td>
</tr>
<tr>
<td>10 ( \mu \text{M} )</td>
<td>15</td>
<td>(-6.3 \pm 2.4^*)</td>
<td>(36.9 \pm 7.6^*)</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SE; \( n \), no. of myocytes in a sample. CPA, cyclopiazonic acid. Caffeine (5 mM) was applied for 5 s. \([\text{Ca}^{2+}\text{]i}\), decrease was composed of 2 successive linear and exponential phases described by the following equations as a function of time (\( t \)):

\[
\text{[Ca}^{2+}\text{]i} = \text{A}(t) + b \quad \text{[Ca}^{2+}\text{]i}_0 + Be^{-\gamma t}
\]

for phases 1 and 2, respectively, where \( A \) is the slope coefficient of phase 1, \( B \) is the peak \([\text{Ca}^{2+}\text{]i}\), value for phase 1, \( \gamma \) is the \([\text{Ca}^{2+}\text{]i}\), value at the onset of phase 2, \( \tau \) is the time constant of phase 2, and \([\text{Ca}^{2+}\text{]i}_0\) is the \([\text{Ca}^{2+}\text{]i}\), value at the end of phase 2. \( *P < 0.05 \) compared with control.

decrease in the percentage of cells generating \([\text{Ca}^{2+}\text{]i}\), oscillations. Indeed, in responding MPA myocytes from CH rats, the pattern of the \([\text{Ca}^{2+}\text{]i}\), response to the agonists acting on seven-transmembrane-domain G-coupled receptors was modified. Although in MPA myocytes from normoxic rats, agonists induce \([\text{Ca}^{2+}\text{]i}\), oscillations that are mainly dependent on a cyclic release of \([\text{Ca}^{2+}\text{]i}\), from an internal store, e.g., the SR (13, 15, 20), in MPA myocytes from CH rats, we observed that ET-1, ANG II, and ATP induced nonoscillating \([\text{Ca}^{2+}\text{]i}\), responses. This change was not due to a CH-induced change in the \([\text{Ca}^{2+}\text{]i}\), sources implicated in the \([\text{Ca}^{2+}\text{]i}\), responses. As in control conditions, agonist-induced \([\text{Ca}^{2+}\text{]i}\), responses in myocytes from CH rats were al - tered neither in a \([\text{Ca}^{2+}\text{]i}\), free solution nor in the presence of the voltage-dependent \([\text{Ca}^{2+}\text{]i}\), channel blocker D-600 but vanished after pretreatment of the cells with thapsigargin (Fig. 3), indicating that they also involved the mobilization of an intracellular \([\text{Ca}^{2+}\text{]i}\), source, presum - ably the SR. This change in the oscillating nature of the agonist-induced \([\text{Ca}^{2+}\text{]i}\), response could be due to either an alteration in the functioning of the Ins(1,4,5)\( P_3 \) receptor and/or to a change in the subtype of Ins(1,4,5)\( P_3 \) receptor involved in the response. In smooth muscle, the Ins(1,4,5)\( P_3 \) receptor is biphasi-
obtained at the beginning of the experiments. Values are means ± SE; n = 5 normoxic and 6 CH rats. Contraction cumulative concentration-response curves for the action of ANG II on rings obtained from control (normoxic) rats and CH rats. Fig. 6. Effect of CH on isometric contraction of MPA rings. A: cumulative concentration-response curves for the action of ET-1 on rings obtained from control (normoxic) rats and CH rats. Contraction is expressed as a percentage of the KCl (80 mM)-induced response obtained at the beginning of the experiments. Values are means ± SE; n = 5 normoxic and 6 CH rats. C: effect of CH on D-600- and TG-resistant component of the contraction induced by ET-1 (100 nM). Contraction is expressed as a percentage of ET-1-induced contraction in the absence of D600 and TG pretreatment.

cally regulated by the [Ca$^{2+}$]$_i$ value (21), and this regulation accounts for the cyclic opening and closure of the associated Ca$^{2+}$ channel (16) and, at least in part, for the so-called Ca$^{2+}$ oscillations (40). It is unlikely that the amplitude of the CH-induced increase in the resting [Ca$^{2+}$]$_i$ value, which averaged 60% but corresponded to an actual value far below 300 μM (21), could have modified the negative feed back of [Ca$^{2+}$]$_i$ on the Ins(1,4,5)P$_3$ receptor. In smooth muscle as in nonmuscle cells, the Ins(1,4,5)P$_3$ receptor is encoded by three different genes, resulting in three isoforms, type 1, type 2, and type 3 Ins(1,4,5)P$_3$ receptors. Recent studies performed on Ins(1,4,5)P$_3$ receptors reconstituted in a lipid bilayer have revealed important functional differences between the three isoforms. Interestingly, only one type of Ins(1,4,5)P$_3$ receptor, type 1, is the isoform that exhibits biphasic regulation by intracellular Ca$^{2+}$ (16). It is thus tempting to speculate that CH could switch the Ins(1,4,5)P$_3$ receptor from a biphasically to a nonbiphasically regulated subtype. This hypothesis requires further molecular biological investigations. Alternatively, this change in the oscillating nature of the agonist-induced [Ca$^{2+}$]$_i$ response could be due to an alteration in the SR Ca$^{2+}$ reuptake mechanisms that play an important role in Ca$^{2+}$ oscillation generation (40). Such Ca$^{2+}$ reuptake mechanisms can be examined by analyzing the falling part of the transient caffeine-induced [Ca$^{2+}$]$_i$ response. First, CH did not modify the percentage of cells responding to caffeine, showing that it only interferes with the Ins(1,4,5)P$_3$-sensitive Ca$^{2+}$ release pathway and not with the caffeine- or ryanodine-sensitive Ca$^{2+}$ release mechanism (Ca$^{2+}$-induced Ca$^{2+}$ release). Nevertheless, the recovery of the resting [Ca$^{2+}$]$_i$ value after caffeine stimulation was clearly slowed down in MPA myocytes from CH rats. Mathematical fitting of the two phases of this recovery showed a significant decrease in the rate of restoration of the resting [Ca$^{2+}$]$_i$ value, suggesting that CH alters the mechanisms of reuptake of Ca$^{2+}$ into the SR, e.g., SR Ca$^{2+}$ pump [sarc(endo)-plasmic reticulum Ca$^{2+}$-ATPase (SERCA)] or/and extrusion of Ca$^{2+}$ [plasmalemma Ca$^{2+}$ pump (plasma membrane Ca$^{2+}$-ATPase) and Na$^+$-Ca$^{2+}$ exchange]. In the present study, the effect of CH on the kinetics of caffeine-induced [Ca$^{2+}$]$_i$ response was mimicked by CPA (Fig. 5), a specific inhibitor of SERCA in this tissue (11). This finding suggests that CH acts at the site of the SR Ca$^{2+}$ pump to delay the recovery of the resting [Ca$^{2+}$]$_i$ value. Interestingly, it has been shown in cardiac muscle, including from humans, that SERCA expression changes during cardiac hypertrophy (2). We believe that this phenomenon accounts for the decrease in the number or disappearance of Ca$^{2+}$ oscillations in responding cells to agonists acting at a membrane receptor. After the first Ca$^{2+}$ increase, Ca$^{2+}$ reuptake into the SR is slowed down, and hence the [Ca$^{2+}$]$_i$ value remains elevated, thus modifying, in turn, the functioning of the Ins(1,4,5)P$_3$ Ca$^{2+}$ release channel.

CH-induced disappearance of Ca$^{2+}$ oscillations in MPA myocytes may play a major role in CH-induced changes in MPA reactivity. Maximal ET-1- and ANG II-induced contraction was decreased as was the frequency of Ca$^{2+}$ oscillations in response to the same agonists, whereas the amount of mobilized Ca$^{2+}$ during the agonist-induced [Ca$^{2+}$]$_i$ response remained unchanged in responding cells. Roux et al. (36) have previously demonstrated that rather than the amount of Ca$^{2+}$, the frequency of Ca$^{2+}$ oscillations plays a critical role in determining the amplitude of the maximal agonist-induced contraction in airway smooth muscle cells. Interestingly, Belouchi et al. (5) have recently shown that CH, which increases the sensitivity of the tracheal contractile response to cholinergic agonists, also increases the Ca$^{2+}$ oscillation frequency in airway smooth muscle myocytes. In this latter smooth muscle, the alteration in contractility was also related to an alteration in Ca$^{2+}$ signaling, although the overall effect was the opposite of that in pulmonary
vascular smooth muscle. The reason for the differential effect of CH on the reactivity of the two smooth muscle types remains to be established.

The results of the present study indicate that CH decreases MPA reactivity via an additional mechanism, i.e., a decrease in the Ca$^{2+}$ sensitivity of the contractile apparatus. This hypothesis is supported by the observations that 1) the D-600- and thapsigargin-resistant component of the contraction was decreased by $\sim$40% in MPA rings from CH rats compared with that in control rats (Fig. 6); under these experimental conditions, agonists do not increase [Ca$^{2+}$]$_i$ in MPA myocytes from either normoxic (13, 15, 20) or hypoxic rats (Fig. 3); and 2) CH had no effect on the contraction induced by an agonist that does not activate a membrane receptor (i.e., KCl). Therefore, the D-600- and thapsigargin-resistant component of the observed contraction is due to a sensitization of the contractile apparatus to Ca$^{2+}$ (39, 40), and CH significantly decreases this sensitization. Interestingly, this latter present result observed in MPAs is opposite to that very recently suggested in intrapulmonary arteries (42). Both results do not indicate the specific pathway among the multiple ones implicated in the Ca$^{2+}$ sensitivity of the smooth muscle contractile apparatus (39) targeted by CH. Again, as discussed above for airway smooth muscle (5), it appears that the effect of CH may vary depending not only on the type of smooth muscle but also on the site along the pulmonary vascular bed. It is also noteworthy that CH also diminishes the response to acute hypoxia in both the pulmonary artery (28) and cardiac muscle (44).

In conclusion, the present study indicates that CH alters pulmonary vascular smooth muscle reactivity as a consequence of an effect on both Ca$^{2+}$ signaling and Ca$^{2+}$ sensitivity of the contractile apparatus. A Ca$^{2+}$ reuptake mechanism appears as a CH-sensitive phenomenon that may account for the main effect of CH on Ca$^{2+}$ signaling, i.e., the loss of agonist-induced Ca$^{2+}$ oscillations.

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