J Appl Physiol
90: 2094–2100, 2001.

Effects of hypercapnia and hypocapnia on $[Ca^{2+}]_i$ mobilization in human pulmonary artery endothelial cells

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Received 26 September 2000; accepted in final form 27 December 2000

Nishio, Kazumi, Yukio Suzuki, Kei Takeshita, Takuya Aoki, Hiroyasu Kudo, Nagato Sato, Katsuhiko Naoki, Naoki Miyao, Makoto Ishii, and Kazuhiro Yamaguchi. Effects of hypercapnia and hypocapnia on $[Ca^{2+}]_i$ mobilization in human pulmonary artery endothelial cells. *J Appl Physiol* 90: 2094–2100, 2001.—The hydrogen ion is an important factor in the alteration of vascular tone in pulmonary circulation. Endothelial cells modulate vascular tone by producing vasoactive substances such as prostacyclin (PGI_2) through a process depending on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We studied the influence of CO_2-related pH changes on $[Ca^{2+}]_i$ and PGI_2 production in human pulmonary artery endothelial cells (HPAECs). Hypercapnic acidosis appreciably increased $[Ca^{2+}]_i$ from 112 ± 24 to 157 ± 38 nmol/l. Intracellular acidification at a normal extracellular pH increased $[Ca^{2+}]_i$ comparable to that observed during hypercapnic acidosis. The hypercapnia-induced increase in $[Ca^{2+}]_i$ was unchanged by the removal of Ca^{2+} from the extracellular medium or by the depletion of thapsigargin-sensitive intracellular Ca^{2+} stores. Hypercapnic acidosis may thus release Ca^{2+} from pH-sensitive but thapsigargin-insensitive intracellular Ca^{2+} stores. Hypocapnic alkalosis caused a fivefold increase in $[Ca^{2+}]_i$ compared with hypercapnic acidosis. Intracellular alkalinization at a normal extracellular pH did not affect $[Ca^{2+}]_i$. The hypocapnia-evoked increase in $[Ca^{2+}]_i$ was decreased from 242 ± 56 to 50 ± 32 nmol/l by the removal of extracellular Ca^{2+}. The main mechanism affecting the hypocapnia-dependent $[Ca^{2+}]_i$ increase was thought to be the augmented influx of extracellular Ca^{2+} mediated by extracellular alkalosis. Hypercapnic acidosis caused little change in PGI_2 production, but hypocapnic alkalosis increased it markedly. In conclusion, both hypercapnic acidosis and hypocapnic alkalosis increase $[Ca^{2+}]_i$ in HPAECs, but the mechanisms and pathophysiological significance of these increases may differ qualitatively.

hypercapnic acidosis; hypocapnic alkalosis; intracellular calcium; PGI_2

INTRACELLULAR AND EXTRACELLULAR pH vary in response to respiratory or metabolic impairment. In pulmonary circulation, pH is an essential factor for changes in vascular tone. The literature indicates that acidosis evokes vasoconstriction, whereas alkalosis causes vasodilatation in pulmonary circulation (12, 16, 17). In fact, hypocapnic alkalosis has been widely used to treat severe neonatal and pediatric pulmonary hypertension (4). Previous studies at our laboratory suggest that changes in the diameter of pulmonary vessels under conditions with abnormal partial pressures of CO_2 (P_{CO_2}) are chiefly mediated by cyclooxygenase (COX)-associated vasoactive substances including prostacyclin (PGI_2) (22, 30). Although COX is known to be mainly expressed in the endothelial cells of pulmonary circulation and requires an increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) for its activation in producing PGI_2 (8, 19), studies of the essential aspects of Ca^{2+} mobilization within pulmonary endothelial cells in response to CO_2-related environmental pH changes caused by hypercapnic acidosis and hypocapnic alkalosis have been few. In the present study, we attempted to assess the effects of hypercapnic acidosis and hypocapnic alkalosis on $[Ca^{2+}]_i$ mobilization in human pulmonary artery endothelial cells (HPAECs) by using fluorescent probes of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) for intracellular pH (pH_i) and fura 2 for $[Ca^{2+}]_i$ indicators. We also studied the significance of Ca^{2+} mobilization from the endoplasmic reticulum and extracellular Ca^{2+} influx for $[Ca^{2+}]_i$ changes in response to hypercapnic acidosis and hypocapnic alkalosis. Finally, we estimated the extent of PGI_2 production in HPAECs that was mediated by hypercapnic acidosis and hypocapnic alkalosis.

MATERIALS AND METHODS

Endothelial cell culture. Passage 4 HPAECs were purchased (Kurabou, Osaka, Japan) and grown to confluence to *passage 7* before experimentation. Thus *passage 8* HPAECs were used for analysis in the present study. HPAECs were cultured on coverslips (Matsunami, Tokyo, Japan) for measurement of $[Ca^{2+}]_i$ and pH_i. We used Humedia-II (Kurabou) containing 10% fetal calf serum, 10 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY) as the culture medium, which was equilibrated in a humidified atmo-

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sphere with 5% CO_2 at 37°C. All measurements were conducted with confluent cell monolayers.

$[Ca^{2+}]_i$ analysis. Transitional changes in $[Ca^{2+}]_i$ were measured with fura 2 as an appropriate indicator (14). Cultured cells were incubated with 2 μM fura 2-AM (Dojin Chemical, Kumamoto, Japan) and 0.08% Pluronic F-127 (Molecular Probes, Eugene, OR) in a standard isotonic solution adjusted to pH 7.4 (in mmol/l: 130 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 1.0 NaH_2PO_4, and 26 HEPES) for 30 min at 37°C. After completion of dye loading, coverslips were washed three times with standard isotonic solution. Dye loading did not change HPAEC morphologies as assessed by light microscopy. Coverslips with fura 2-loaded cells were placed in a flow chamber and mounted on the stage of a microspectrometer connected to a fluorescence microscope (Diaphot T300, Nikon, Tokyo, Japan) with an excitation filter changer and a multipoint imaging system (Argus 50, Hamamatsu Photonics, Shizuoka, Japan). Coverslips were perfused with warmed buffer at 2 ml/min; the temperature of the measurement system was maintained at 37°C. After 20 min of perfusion, cells were alternately excited at two wavelengths, 340 and 380 nm, and resultant light emission was detected at 510 nm. Fluorescence intensity data, F_{340} and F_{380}, were analyzed using customized software. The ratio (R) of fura 2 fluorescence intensities excited by two wavelengths (F_{340}/F_{380}) was calculated after subtraction of background fluorescence at every 15 s. Maximum and minimum fluorescence ratios (R_{max} and R_{min}) were determined by using 10 mmol/l of $CaCl_2$ and EGTA (Dojin Chemical), respectively. $[Ca^{2+}]_i$ was calculated based on a formula proposed by Grynkiewicz et al. (14)

$$[Ca^{2+}]_i = (R - R_{min})/(R_{max} - R) \times \beta \times K_d$$

where β is the ratio of emission fluorescence at 380 nm in the presence of 10 mmol/l $CaCl_2$ and EGTA. Measured β values averaged 12.4 in our Ca^{2+} measurement system ($n = 5$). Because the dissociation constant (K_d) of fura 2 with Ca^{2+} is significantly affected by pH_i changes, it was corrected with the following equation (5)

$$K_d = 224 \times \{1/[3.1 \times (pH_i - 5.77)] + 0.73\}$$

pH_i analysis. pH_i was measured with BCECF, a fluorescent pH indicator dye (23). BCECF-AM (Molecular Probes) was dissolved in DMSO initially at 2.5 mmol/l and was used at a final concentration of 5 μmol/l in standard isotonic solution. Cells were loaded with BCECF for 30 min at 37°C. To measure pH_i, we applied the excitation wavelengths of 490 and 450 nm and measured emission at 530 nm. The ratio of fluorescence intensity (F_{490}/F_{450}) was used to assess the change in pH_i. pH_i measurement was calibrated with nigericin (7 μmol/l) containing high-K^+ solution (40 mmol/l) at different extracellular pH (pH_o) levels. Fluorescence ratio data thus obtained were analyzed by linear regression to calculate pH_i (26). The pH_i was measured at intervals of 15 s, thus allowing us to estimate the K_d value at every 15 s.

Hypercapnic acidosis and hypocapnic alkalosis. Cells were perfused at 2 ml/min on the microscope stage for 20 min with Krebs-Henseleit buffer solution (in mmol/l: 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_47H_2O$, 2.5 $CaCl_26H_2O$, 25 $NaHCO_3$, and 5.6 glucose) equilibrated with a gas mixture containing 21% O_2 and 5% CO_2. P_{CO_2} in the circulating buffer was changed by switching the equilibration gas from normocapnia (21% O_2 and 5% CO_2 in N_2) to either hypercapnia (21% O_2 and 10% CO_2 in N_2, $n = 10$) or hypocapnia (21% O_2 and 2% CO_2 in N_2, $n = 10$). Equilibration by hypercapnic gas and hypocapnic gas changed the pH_o from 7.4 to 7.0 and from 7.4 to 7.8, respectively. We examined the transitional changes in pH_i and $[Ca^{2+}]_i$ at every 15 s under each experimental condition.

Intracellular acidification and alkalinization. To determine the importance of intracellular acidification in the modulation of $[Ca^{2+}]_i$ in hypercapnic acidosis, endothelial cells were perfused with physiological HEPES buffer solution (in mmol/l: 137 NaCl, 5.0 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.2 $CaCl_2$, 10 HEPES, and 16 glucose) in which pH was adjusted to 7.4, and then the circulating medium was switched to Krebs-Henseleit solution equilibrated with a gas mixture containing 21% O_2 and 5% CO_2, enabling pH_o to be maintained at 7.4 ($n = 7$).

To determine the role of intracellular alkalinization in modulating $[Ca^{2+}]_i$ on hypocapnic alkalosis, endothelial cells were first perfused with physiological HEPES buffer solution ($pH_o = 7.4$). The perfusate was then changed to HEPES buffer containing 20 mmol/l NH_4Cl in which pH was adjusted to 7.4 by equilibrating the perfusate with room air containing no CO_2 ($n = 6$). Under these experimental conditions, we measured changes in pH_i and $[Ca^{2+}]_i$ at intervals of 15 s.

Contribution of extracellular Ca^{2+} and intracellular Ca^{2+} stores. We studied Ca^{2+} sources involved in pH-dependent $[Ca^{2+}]_i$ mobilization in HPAECs. To determine the relative contribution of extracellular Ca^{2+}, pH-induced $[Ca^{2+}]_i$ changes were measured in Ca^{2+}-free Krebs-Henseleit solution containing 2 mmol/l of EGTA in hypercapnic acidosis ($n = 6$) and hypocapnic alkalosis ($n = 8$). To analyze the importance of intracellular Ca^{2+} stores, extracellular and intracellular Ca^{2+} stores were depleted simultaneously. Extracellular stores were decreased by EGTA and intracellular stores by introducing 100 nmol/l of thapsigargin (TG) (Wako, Osaka, Japan). Under these experimental conditions, we calculated $[Ca^{2+}]_i$ changes as a result of hypercapnic acidosis ($n = 6$) and hypocapnic alkalosis ($n = 6$).

To validate the efficiency of EGTA and TG, we preliminarily examined histamine-elicited $[Ca^{2+}]_i$ changes in HPAECs in the presence of these agents. Histamine has been shown to induce not only an early-phase transient increase in $[Ca^{2+}]_i$ by Ca^{2+} release mainly from intracellular stores but also a late-phase plateau by augmenting Ca^{2+} influx from the extracellular medium (21). Histamine transiently increased $[Ca^{2+}]_i$ from 105 ± 18 to 583 ± 146 nmol/l (means ± SE) in the absence of EGTA and TG ($n = 3$). This augmented $[Ca^{2+}]_i$ gradually decreased and reached the plateau at 178 ± 32 nmol/l. In the presence of EGTA, the application of histamine yielded the early-phase transient increase in $[Ca^{2+}]_i$ from 98 ± 12 to 360 ± 107 nmol/l, followed by a continuous decrease in $[Ca^{2+}]_i$ that did not reach the plateau ($n = 3$), thus confirming the efficacy of EGTA chelating extracellular Ca^{2+} because the late-phase plateau of $[Ca^{2+}]_i$ mediated by Ca^{2+} entry from the extracellular medium disappeared. Histamine-elicited $[Ca^{2+}]_i$ kinetics investigated in the presence of both EGTA and TG revealed that histamine had no significant influence on the $[Ca^{2+}]_i$ in HPAECs, i.e., $[Ca^{2+}]_i$ changed from 116 ± 19 to 124 ± 23 nmol/l, with no difference between the two ($n = 3$). These findings indicate that the efficacy of TG depleting intracellular Ca^{2+} stores is sufficiently high because of the disappearance of the early-phase increase in $[Ca^{2+}]_i$ caused by Ca^{2+} release from intracellular stores.

PGI_2 measurement. HPAECs were cultured in 25-cm^2 tissue culture flasks and used at confluence. Experiments were conducted in a warm dish at 37°C. The culture medium was replaced with Krebs-Henseleit solution equilibrated with gas

containing 5% CO_2 and 21% O_2 (pH_o = 7.4). After 1 h, the supernatant was removed and another 3 ml of Krebs-Henseleit buffer were equilibrated with normocapnic, hypercapnic, or hypocapnic gas; a gas mixture having the same gas composition as used for the buffer equilibration was continuously supplied to the dish. The pH of each supernatant was maintained at 7.38 ± 0.03 (normocapnia, n = 5), 7.01 ± 0.02 (hypercapnia, n = 5), or 7.82 ± 0.04 (hypocapnia, n = 5). Ten minutes later, 0.5 ml of the supernatant was removed to measure PGI_2. All samples were stored at −20°C, and 6-keto prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), the stable hydrolysis product of PGI_2, was measured by radioimmunoassay (18).

Statistical analysis. Data are presented as means ± SE. We judged the statistical significance of the changes in pH_i and $[Ca^{2+}]_i$ when the gas mixture was altered from normocapnia to hypercapnia or hypocapnia by the paired t-test. Differences in pH_i and $[Ca^{2+}]_i$ before and after intracellular acidification or alkalinization was introduced were also judged by the paired t-test. Statistical differences in increments of $[Ca^{2+}]_i$ in hypercapnic acidosis and in hypocapnic alkalosis were determined by the unpaired t-test. Changes in $[Ca^{2+}]_i$ among groups with different medications were compared by applying ANOVA followed by Scheffé's F test. Differences in PGI_2 production among normocapnia, hypercapnia, and hypocapnia were also judged by ANOVA and Scheffé's F test. A P value of <0.05 was considered statistically significant.

RESULTS

Effect of hypercapnia and hypocapnia on $[Ca^{2+}]_i$. Exposure to hypercapnic acidosis rapidly decreased pH_i from 7.19 ± 0.07 to 7.04 ± 0.11, in association with a modest increase in $[Ca^{2+}]_i$ from 112 ± 24 to 157 ± 38 nmol/l (Fig. 1). Exposure to hypocapnic alkalosis increased pH_i from 7.18 ± 0.05 to 7.34 ± 0.10, leading to a marked increase in $[Ca^{2+}]_i$ from 114 ± 18 to 359 ± 72 nmol/l. $\Delta[Ca^{2+}]_i$-to-ΔpH_i ratios (where Δ means change) were much larger for hypocapnic alkalosis [(15 ± 4) × 10^2] than hypercapnic acidosis [(3.0 ± 0.7) × 10^2].

Effect of intracellular acidification and alkalinization on $[Ca^{2+}]_i$. Intracellular acidification (ΔpH_i = −0.17 ± 0.03) at pH_o maintained at 7.4 significantly augmented $[Ca^{2+}]_i$ by 24 ± 8 nmol/l, whereas intracellular alkalinization (ΔpH_i = +0.38 ± 0.08) at a constant pH_o of 7.4 had little influence on $[Ca^{2+}]_i$ (Fig. 2). The increase in $[Ca^{2+}]_i$ elicited by intracellular acidification did not differ from that obtained under conditions with hypercapnic acidosis.

Contribution of extracellular Ca^{2+} and TG-sensitive intracellular Ca^{2+} pool to the increase in $[Ca^{2+}]_i$. Hypercapnic acidosis increased $[Ca^{2+}]_i$ by 45 ± 14 nmol/l under control conditions in the absence of EGTA and TG, by 40 ± 13 nmol/l in the presence of EGTA (removal of Ca^{2+} from the perfusate), and by 35 ± 16 nmol/l in the presence of both EGTA and TG (depletion of both extracellular and intracellular Ca^{2+}) (Fig. 3). There was no significant difference between the values.

Hypocapnic alkalosis enhanced $[Ca^{2+}]_i$ by 242 ± 56 nmol/l under control conditions in the absence of medication, whereas the removal of extracellular Ca^{2+} by

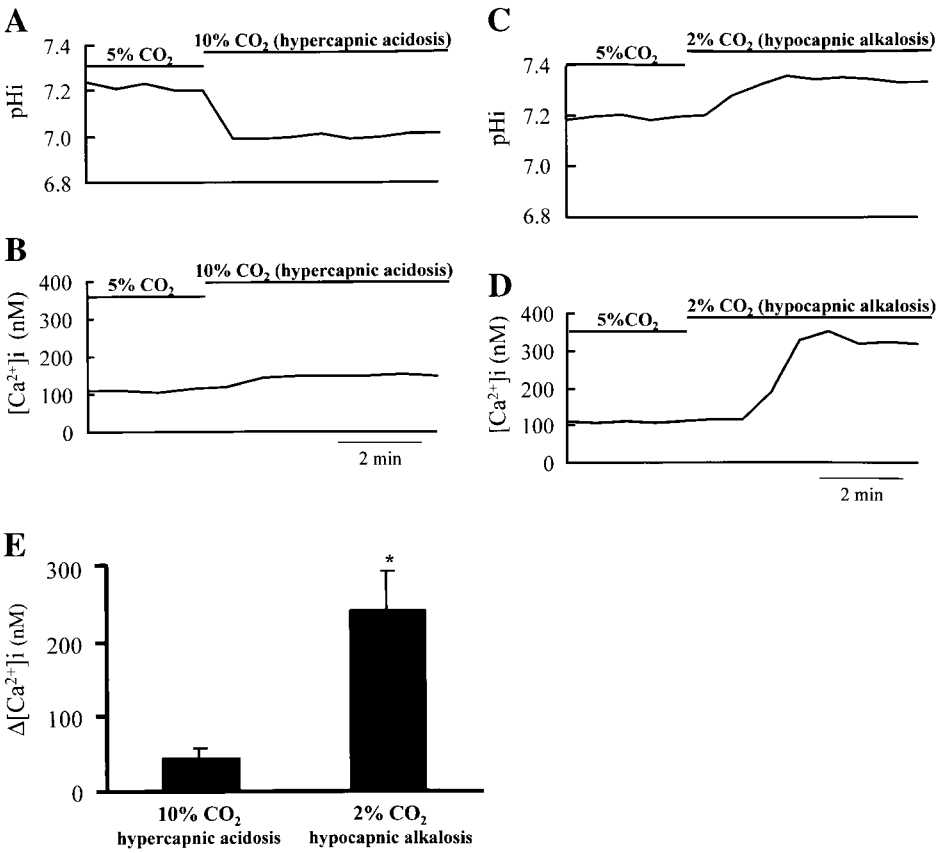

Fig. 1. Examples of intracellular pH (pH_i) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) changes in hypercapnic acidosis and hypocapnic alkalosis. *A*: pH_i changes in hypercapnic acidosis. *B*: $[Ca^{2+}]_i$ changes in hypercapnic acidosis. *C*: pH_i changes in hypocapnic alkalosis. *D*: $[Ca^{2+}]_i$ changes in hypocapnic alkalosis. The increase in $[Ca^{2+}]_i$ in hypocapnic alkalosis was significantly greater than that in hypercapnic acidosis (*E*). *$P < 0.05$ compared with the values obtained in hypercapnic acidosis.

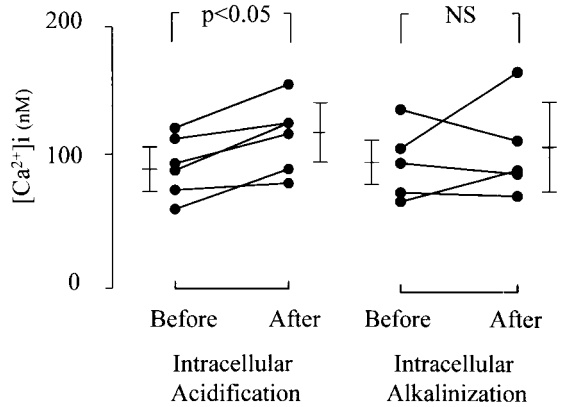

Fig. 2. Effects of intracellular acidification and alkalinization on $[Ca^{2+}]_i$ in human pulmonary artery endothelial cells (HPAECs). Intracellular acidification increased $[Ca^{2+}]_i$, but intracellular alkalinization did not. NS, not significant.

EGTA drastically reduced the extent of hypocapnia-induced $[Ca^{2+}]_i$ increase (50 ± 32 nmol/l). Depletion of both extracellular Ca^{2+} and intracellular Ca^{2+} stores by EGTA and TG further inhibited the increment of $[Ca^{2+}]_i$ (12 ± 30 nmol/l).

Effect of hypercapnia and hypocapnia on PGI_2 production. Hypercapnic acidosis did not augment endothelial PGI_2 production compared with that obtained under normocapnic conditions (Fig. 4). However, hypocapnic alkalosis markedly augmented PGI_2 production in pulmonary artery endothelial cells.

DISCUSSION

Critique of methods. In the present study, we used *passage 8* HPAECs for assessing the relationship between pH_i and $[Ca^{2+}]_i$ under various experimental conditions. To see whether differences in the passage of HPAECs would exert a significant influence on $[Ca^{2+}]_i$ mobilization, we preliminarily examined the hypercapnia- and hypocapnia-elicited $[Ca^{2+}]_i$ kinetics in HPAECs ranging from *passage 8* to *passage 12*. $[Ca^{2+}]_i$ kinetics observed for these HPAECs with various passages did not differ quantitatively, indicating that the difference in passage would have little effect on $[Ca^{2+}]_i$ mobilization evoked by hypercapnic acidosis and hypocapnic alkalosis.

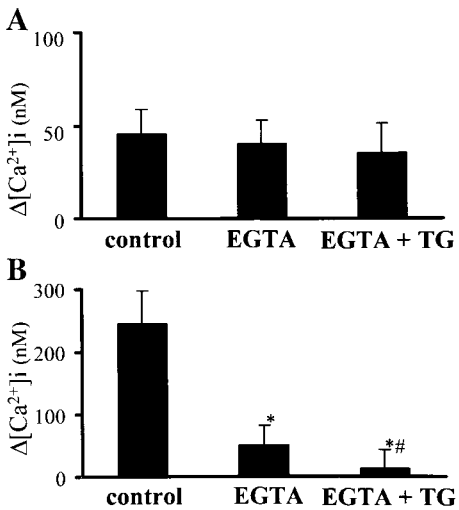

Fig. 3. Contribution of extracellular Ca^{2+} and thapsigargin (TG)-sensitive Ca^{2+} stores to the increase in $[Ca^{2+}]_i$ with hypercapnic acidosis and hypocapnic alkalosis. *A*: hypercapnia-induced $[Ca^{2+}]_i$ changes in the presence of EGTA only or both EGTA and TG. Removal of Ca^{2+} from the perfusate by EGTA and depletion of intracellular Ca^{2+} stores by TG did not significantly affect the hypercapnia-induced $[Ca^{2+}]_i$ increase. *B*: hypocapnia-induced $[Ca^{2+}]_i$ changes in the presence of EGTA only or both EGTA and TG. Treatment with both EGTA and TG almost abolished the $[Ca^{2+}]_i$ increase. $^*P < 0.05$ compared with the control group. $\#P < 0.05$ compared with the EGTA group.

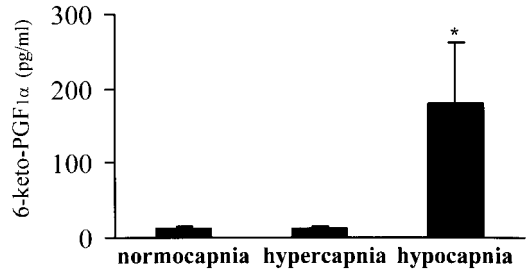

Fig. 4. Effect of hypercapnic acidosis and hypocapnic alkalosis on 6-keto-$PGF_{1\alpha}$ production in HPAECs. Hypercapnia did not increase 6-keto-$PGF_{1\alpha}$ production, whereas hypocapnia increased it significantly. $^*P < 0.05$ compared with normocapnia.

Although we estimated the absolute value of $[Ca^{2+}]_i$ based on the formula reported by Grynkiewicz et al. (14), there may be a couple of limitations with this formula. The first limitation is the linearity between R_{max} (fura 2 signal saturated with Ca^{2+}) and R_{min} (fura 2 signal in the absence of Ca^{2+}). This issue was extensively addressed by Grynkiewicz et al. (14), who confirmed the linearity of fura 2 fluorescence intensities at 340 and 380 nm in the presence of Ca^{2+} raging from 0 to 100 mmol/l. The other crucial point in the Grynkiewicz formula is that the K_d of fura 2 with Ca^{2+} is considerably affected by environmental ionic strength, temperature, and pH. Because ionic strength within HPAECs is expected to be approximately constant and the temperature was certainly maintained at 37°C during all the measurements, the effect of variations in pH_i on K_d may be particularly important in the present study. Based on these facts, we estimated the K_d value corresponding to a given pH_i by referring to the formula reported by Batlle et al. (5), who determined the relationship between pH_i and K_d.

We used Krebs-Henseleit solution for hypercapnic and hypocapnic experiments but physiological HEPES buffer for the experiments aimed at intracellular acidification and alkalinization. We encountered great difficulties in establishing a sustained decrease or increase in pH_i in Krebs-Henseleit solution under conditions in which pH_o was adjusted to 7.4 by equilibrating the solution with a gas mixture containing an appropriate fraction of CO_2. Because we could not exclude the possibility that the differences in composition in extracellular media would exert an appreciable influence on the results of $[Ca^{2+}]_i$, we preliminarily compared the $[Ca^{2+}]_i$ changes mediated by intracellular alkalosis produced in physiological HEPES buffer

with NH_4Cl and those in Krebs-Henseleit solution with a small amount of $NaHCO_3$ equilibrated with a gas containing a low concentration of CO_2. These experimental conditions established a comparable level of intracellular alkalosis at pH_o adjusted to 7.4. Intracellular alkalinization was selected for comparison because it was somewhat easier to produce the stable level of intracellular alkalosis than that of intracellular acidosis in Krebs-Henseleit solution in which pH_o was maintained at 7.4. We found little statistical difference in $[Ca^{2+}]_i$ kinetics between the two conditions, leading us to believe that the difference in compositions in extracellular media might not significantly affect $[Ca^{2+}]_i$ kinetics.

Differential effects of hypercapnic acidosis and hypocapnic alkalosis on intracellular Ca^{2+} mobilization in endothelial cells. Although many studies have focused on the significance of acidosis or alkalosis in Ca^{2+} mobilization in vascular endothelial cells, most of them addressed aortic endothelial cells rather than pulmonary endothelial cells (9, 11, 25, 27, 28, 32). Furthermore, findings obtained for aortic endothelial cells are mutually qualitatively inconsistent. For instance, Ziegelstein et al. (32) found that intracellular acidification enhanced $[Ca^{2+}]_i$, whereas intracellular alkalinization reduced it in cultured rat aortic endothelial cells. On the other hand, Danthuluri et al. (9) reported findings opposing those of Ziegelstein et al., suggesting that intracellular alkalinization increased Ca^{2+} in bovine aortic endothelial cells. In regard to which one is important in mobilizing Ca^{2+} (pH_i or pH_o), some researchers demonstrated that the reduction of pH_o, but not pH_i, was the essential factor regulating $[Ca^{2+}]_i$ in canine coronary artery endothelial cells subjected to severe acidosis (11, 25). These researchers also showed that intracellular alkalinization increased $[Ca^{2+}]_i$ in bovine aortic endothelial cells (11, 25). On the other hand, Wakabayashi and Groschner (28) reported that the extracellular elevation of pH was the primary cause increasing $[Ca^{2+}]_i$ in vascular endothelial cell line ECV 304. Experimental findings reported in the literature thus do not provide a confident conclusion on the regulatory mechanism of $[Ca^{2+}]_i$ mobilization by pH even in endothelial cells originating from systemic circulation. No study has approached the interrelationship between pH_o and/or pH_i environments and $[Ca^{2+}]_i$ regulation in pulmonary endothelial cells, which have functions that are expected to be qualitatively different from those of endothelial cells in systemic circulation (22, 30). Nitric oxide synthase and COX expressed in endothelial cells are equivalently important enzymes for regulating vascular tone in systemic circulation in response to acidosis and alkalosis (1). Endothelial COX, however, appears to be much more important than endothelial nitric oxide synthase in maintaining vascular tone in pulmonary circulation when environmental pH is changed (22, 30). Based on these facts, we systematically analyzed the quantitative effects of acidosis and alkalosis implemented by changing CO_2 concentrations (i.e., hypercapnic acidosis and hypocapnic alkalosis) on $[Ca^{2+}]_i$ and its effects on yielding COX-related vasoactive substance of PGI_2 in pulmonary endothelial cells originally obtained from human pulmonary arteries. P_{CO_2} varies significantly from region to region even in normal lungs (29). The regional variation of P_{CO_2} modifies the constrictive state of pulmonary vessels there (13), firmly indicating that it is important to analyze the mechanisms involved in regulating $[Ca^{2+}]_i$ under conditions with hypercapnic acidosis and hypocapnic alkalosis in pulmonary endothelial cells.

We found that hypercapnic acidosis slightly but significantly enhanced $[Ca^{2+}]_i$ in HPAECs (Fig. 1). The increment of $[Ca^{2+}]_i$ induced by hypercapnic acidosis was comparable to that elicited by intracellular acidification at a constant pH_o adjusted to 7.4 (Fig. 2). These findings suggest that the hypercapnia-associated increase in $[Ca^{2+}]_i$ is mainly mediated by intracellular acidosis but not by extracellular acidosis or pH gradient across the cell membrane. Although pH gradient differed considerably between hypercapnic acidosis and intracellular acidification, $[Ca^{2+}]_i$ changes were comparable between them, eliminating the importance of pH gradient for $[Ca^{2+}]_i$ kinetics during hypercapnic acidosis. The removal of extracellular Ca^{2+} by EGTA little influenced $[Ca^{2+}]_i$ in hypercapnic acidosis (Fig. 3), indicating that extracellular Ca^{2+} would not play any major role in regulating $[Ca^{2+}]_i$ under acidic conditions with hypercapnia. The simultaneous depletion of extracellular Ca^{2+} by EGTA and intracellular Ca^{2+} stores sensitive to TG did not change the increment of $[Ca^{2+}]_i$ in hypercapnic acidosis (Fig. 3). Because TG is an inhibitor against Ca^{2+}-ATPase and depletes Ca^{2+} in the endoplasmic reticulum (32), the TG experiments suggest that hypercapnic acidosis mobilizes Ca^{2+} either from intracellular binding sites or from TG-insensitive intracellular Ca^{2+} store sites such as the mitochondria.

Interestingly, $[Ca^{2+}]_i$ kinetics in hypocapnic alkalosis were quite different from those observed in hypercapnic acidosis. Although the increment of $[Ca^{2+}]_i$ in hypocapnic alkalosis was distinctly greater than that in hypercapnic acidosis (Fig. 1), intracellular alkalinization established at a constant pH_o had little effect on $[Ca^{2+}]_i$ (Fig. 2), indicating that extracellular alkalosis and/or pH gradient across the cell membrane would be of greater importance in regulating $[Ca^{2+}]_i$ under hypocapnic conditions. The issue of which is important for hypocapnia-induced $[Ca^{2+}]_i$ increase, pH_o itself or pH gradient, is not conclusively settled from the experimental findings obtained in the present study. Further studies are absolutely necessary for clarifying this point. Removal of extracellular Ca^{2+} by EGTA markedly decreased the increment of $[Ca^{2+}]_i$ in hypocapnic alkalosis (Fig. 3), suggesting that, in contrast to hypercapnic acidosis, extracellular Ca^{2+} would be the primary source for Ca^{2+} mobilization in hypocapnic alkalosis. We also found further inhibition of Ca^{2+} mobilization by concomitant treatment with EGTA and TG in hypocapnic alkalosis (Fig. 3). These findings may indicate that TG-sensitive intracellular Ca^{2+} stores also play a role in regulating $[Ca^{2+}]_i$ under hypocapnic

conditions. Considering both types of data obtained for hypercapnic acidosis and hypocapnic alkalosis, the primary mechanism of endothelial Ca^{2+} mobilization is qualitatively different between the two conditions centering pH_o at 7.4 corresponding to pH_i of 7.2, even though the two conditions were established simply by changing CO_2 concentrations in the medium.

Elevation in cytosolic Ca^{2+} in endothelial cells is caused by Ca^{2+} entry via various ion channels in the plasma membrane and by Ca^{2+} release from intracellular stores. Because endothelial cells are lacking in the voltage-gated Ca^{2+} channel (2), their Ca^{2+} entry may be mediated by four different channels: *1*) receptor-mediated channel coupled to second messengers, *2*) Ca^{2+} leak channel dependent on the electrochemical gradient, *3*) stretch-activated nonselective cation channel, and *4*) Na^+-dependent Ca^{2+} entry (i.e., Na^+/Ca^{2+} exchange). Among them, the Ca^{2+} leak channel and the Na^+/Ca^{2+} exchanger may play major roles in regulating endothelial Ca^{2+} entry under physiological conditions in the absence of specific agonists (2). Our experimental findings compulsorily suggested that intracellular elevation in Ca^{2+} under hypercapnic conditions would be mainly mediated by intracellular events associated with intracellular acidosis (Figs. 1 and 2). Intracellular acidosis may activate the Na^+/H^+ exchanger and increase intracellular Na^+, augmenting Ca^{2+} entry from the extracellular medium via the Na^+/Ca^{2+} exchanger. However, this may not explain the enhanced intracellular Ca^{2+} on hypercapnia (Fig. 1) because the depletion of extracellular Ca^{2+} by EGTA did not suppress the increase in intracellular Ca^{2+} during hypercapnia exposure (Fig. 3). Furthermore, participation of a Ca^{2+} leak channel in hypercapnia-related increases in intracellular Ca^{2+} is unlikely because of the same reason as described above. In support of this notion, alkalosis, but not acidosis, has been demonstrated to activate the endothelial Ca^{2+} leak channel (28). Thus we considered that hypercapnia-related increases in intracellular Ca^{2+} might be caused by decreasing affinity of Ca^{2+} to Ca^{2+}-ATPase by acidosis, which would reduce Ca^{2+} uptake into the endoplasmic reticulum, or by increasing Ca^{2+} release from TG-insensitive intracellular stores such as the mitochondria (24). On the other hand, we found that hypocapnia would mainly enhance intracellular Ca^{2+} via increased Ca^{2+} entry from the extracellular medium (Figs. 1 and 2). This may be mostly explained by activation of the Ca^{2+} leak channel by extracellular alkalosis, as proposed by Wakabayashi and Groschner (28).

Physiological significance. To assess the importance of endothelial $[Ca^{2+}]_i$ changes in modulating vascular tone in pulmonary circulation under hypercapnic and hypocapnic conditions, we estimated the effects of hypercapnia and hypocapnia on the production of PGI_2, which is expected to be an essential vasodilating prostaglandin yielded by COX (Fig. 4). As discussed above, we confirmed in previous studies that COX-mediated vasoactive substances play a significant role in modifying pulmonary vascular tone under conditions with varied pH induced by changes in CO_2 concentrations (22, 30). As presumed from the large difference in the increment of $[Ca^{2+}]_i$ (Fig. 1), hypocapnic alkalosis produced PGI_2 about 10 times as much as hypercapnic acidosis (Fig. 4). Enhanced PGI_2 production may support the hypocapnia-elicited vasodilatation consistently observed in pulmonary circulation (4, 12, 13). Several groups of investigators showed that hypercapnic acidosis generally evoked pulmonary vasoconstriction (6, 10, 16, 17, 31), which would not appear to be at variance with a small amount of PGI_2 produced on the stimulation of hypercapnic acidosis observed in the present study. However, the small amount of PGI_2 does not explain the positive vasoconstriction of pulmonary vasculature in hypercapnic acidosis. Ahn and Hume (3) and Berger et al. (7) ascertained that acidosis-elicited constriction of pulmonary arterial smooth muscle cells was mediated through the inhibition of voltage-dependent K^+ channels. Combining our findings with the electrophysiological results reported by the above authors (3, 7), pulmonary vasoconstriction induced by hypercapnic acidosis is attributable to the direct effect of acidosis on smooth muscle cells, whereas the significance of endothelial function modified by acidosis may be trivial for hypercapnia-related pulmonary vasoconstriction.

Another important issue observed in the present study is that endothelial function in pulmonary circulation differs qualitatively from that in systemic circulation, i.e., pulmonary endothelial cells produce a large amount of PGI_2 in response to hypocapnic alkalosis (Fig. 4), but systemic endothelial cells originating in cerebral vessels and the aorta yield PGI_2 in response to hypercapnic acidosis and not to hypocapnic alkalosis (15, 20).

In conclusion, although hypercapnic acidosis and hypocapnic alkalosis are physiologically consecutive conditions, their regulatory effects on $[Ca^{2+}]_i$ in pulmonary artery endothelial cells are not simply explained from a single mechanism. Independent of extracellular Ca^{2+}, hypercapnic acidosis mobilizes a small amount of Ca^{2+} from TG-insensitive intracellular Ca^{2+} stores in pulmonary endothelial cells, leading to no significant production of PGI_2. However, hypocapnic alkalosis markedly increases $[Ca^{2+}]_i$ mainly through the enhanced influx of extracellular Ca^{2+}. Hypocapnic alkalosis therefore produces a large amount of PGI_2 in pulmonary endothelial cells.

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