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Elevated Carbon Dioxide Blunts Mammalian cAMP Signaling Dependent on Inositol 1,4,5-Triphosphate Receptor-mediated Ca^{2+} Release*[S]

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Background: Elevated CO_2 is toxic to mammalian cells.
Results: Molecular CO_2 reduces cellular cAMP dependent on intracellular Ca^{2+}.
Conclusion: CO_2 can alter cellular physiological processes through IP_3-mediated Ca^{2+} release.
Significance: Altered Ca^{2+} signaling mediated by CO_2 might underpin the detrimental effects of CO_2 on the cell.

Elevated CO_2 is generally detrimental to animal cells, suggesting an interaction with core processes in cell biology. We demonstrate that elevated CO_2 blunts G protein-activated cAMP signaling. The effect of CO_2 is independent of changes in intracellular and extracellular pH, independent of the mechanism used to activate the cAMP signaling pathway, and is independent of cell context. A combination of pharmacological and genetic tools demonstrated that the effect of elevated CO_2 on cAMP levels required the activity of the IP_3 receptor. Consistent with these findings, CO_2 caused an increase in steady state cytoplasmic Ca^{2+} concentrations not observed in the absence of the IP_3 receptor or under nonspecific acidotic conditions. We examined the well characterized cAMP-dependent inhibition of the isoform 3 Na^+/H^+ antiporter (NHE3) to demonstrate a functional relevance for CO_2-mediated reductions in cellular cAMP. Consistent with the cellular biochemistry, elevated CO_2 abrogated the inhibitory effect of cAMP on NHE3 function via an IP_3 receptor-dependent mechanism.

The importance of CO_2 in biology is paramount. CO_2 is integral to all life as the substrate for the CO_2-fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco)[2] in photosynthetic organisms and is a substrate/product for many other metabolic enzymes. The pH-dependent CO_2/bicarbonate equilibrium is fundamental to physiology and is intimately associated with homeostatic mechanisms, including pH regulation, volume control, and fluid secretion.

All life on Earth has continued to flourish despite being subjected to large fluctuations in the levels of CO_2 in both the atmosphere and aquatic environments (1). Photosynthetic organisms are able to acclimate to large changes in atmospheric CO_2 (2). Fluctuations in CO_2 can also apply stress to unicellular and multicellular organisms over much shorter time scales. Aquatic environments can show both diurnal and long-term seasonal variations in CO_2 with consequent effects on photosynthetic organisms (3). Increased respiration during exercise can cause the partial pressure of CO_2 rise from 35–45 mm Hg to over 120 mm Hg. Specific mechanisms exist to detect elevated CO_2 and enable appropriate responses, but CO_2 can also have relatively nonspecific deleterious effects on the cell (4).

CO_2 is proposed to enter cells through aquaporin, Amt, and Rhesus channels (5–7) and have direct effects on protein through carbamate formation, for example on Rubisco and hemoglobin (8). About 20 Protein Data Bank structures have CO_2 as a ligand with a variety of modes of interaction but primarily through interactions with basic side chains (9). CO_2 and HCO_3^- also influence a number of cell signaling processes. CO_2 activates fungal pathogenesis through AC, and additional ACs from prokaryotes and mammals also respond directly to CO_2 and HCO_3^- (10–12). HCO_3^- activates guanylyl cyclase types D and G to enable CO_2 olfaction (13–15). The role of the cGMP pathway in CO_2 chemosensing has also been conserved in *Caenorhabditis elegans* avoidance behavior (16–18). Chemosensing of CO_2 in *Drosophila melanogaster* is mediated through *Gr21a* and *Gr63a*, two receptors of the seven-transmembrane domain class (19, 20). In mammals, ATP release is key to chemosensory control of respiration and increased CO_2 permits ATP release from the medulla oblongata through a mechanism that requires connexin 26 (21, 22). CO_2 is also chemosensed by specific taste receptor cells that express carbonic anhydrase type 4 and has evolutionarily conserved inhibitory effects on innate immunity through inhibition of NF-κB signaling (23–26). In acute acid-base disturbance, the proximal tubule cells of the mammalian kidney respond directly to CO_2 to stimulate

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[2] The abbreviations used are: Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; AC, adenylyl cyclase; 2-APB, 2-aminoethoxydiphenyl borate; Fsk, forskolin; Fura 2-AM, Fura-2 pentakis-acetoxymethyl ester; IP_3, inositol 1,4,5-triphosphate; IP_3R, IP_3 receptor; PTH, parathyroid hormone; BCECF-AM, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; OK, opossum kidney.

H^+ secretion through a mechanism involving a tyrosine kinase of the epidermal growth factor receptor family and intracellular Ca^{2+} (27, 28). It is clear that specific mechanisms exist, through which CO_2 can interact with biological systems.

The examples provided are mechanisms by which CO_2 is detected specifically to initiate adaptive physiological responses, but CO_2 can have generally detrimental effects on animal cellular processes. In *C. elegans*, increased CO_2 causes slowed development, reduced fertility, and causes deterioration of body musculature (29). In *Drosophila*, elevated CO_2 causes defects in embryonic development and egg laying and hatching (25). Elevated CO_2 in rats stimulates renal phosphate excretion that is independent of other physiological factors, including pH (30). CO_2 will also impair alveolar fluid reabsorption in alveolar type II epithelial cells by inducing Na^+,K^+-ATPase endocytosis (31, 32). CO_2 can also chronically decrease cell proliferation through increasing levels of the miR-183 microRNA (33). Generally speaking, elevated CO_2 is tolerated in humans, although toxic effects on the central nervous system, cardiovascular, renal, metabolic, and respiratory systems are evident. Despite this, some individuals may show a greater sensitivity to the adverse effects of CO_2, for example, in the presence of an increased intracranial pressure. The significance of this is that ventilation strategies in patients that induce hypercapnia, so-called "permissive hypercapnia," improves prognosis in models of acute lung injury, ischemia-reperfusion injury and acute respiratory distress syndrome (34, 35). The protective effect of permissive hypercapnia is explained to a large extent by the anti-inflammatory influence of CO_2, but such immune suppression may be detrimental in clinical settings where infection or wounding is present (26, 36). There is a requirement, therefore, to understand the molecular basis of CO_2 interactions with the core processes of the cell to understand how CO_2 can be detrimental to cell function across the animal kingdom and also to inform clinical decisions regarding the use of permissive hypercapnia. In direct contrast to the current paradigm where CO_2 can activate specific physiological processes through accumulation of cyclic nucleotides, we demonstrate that CO_2 blunts cellular activities regulated by cAMP. This effect is independent of pH and requires Ca^{2+} release via the IP_3 receptor. Given the ubiquity of cAMP and Ca^{2+} signaling in mammalian cells, this work suggests a key mechanism by which CO_2 can have a broad spectrum of effects on cell physiology independent of pH.

EXPERIMENTAL PROCEDURES

Cell Culture—OK cells (gift of Heini Murer, University of Zurich) and HEK-PR1 cells (gift of Colin Taylor, University of Cambridge) were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's Nutrient Mixture F12 (1:1 volume), 15 mM HEPES, 14 mM $NaHCO_3$, 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin, 2% (v/v) non-essential amino acids, 1% (v/v) L-glutamine, and 500 μg/ml G418 (HEK-PR1 cells only). UMR-106 cells (gift of James Gallagher, University of Liverpool) were cultured in DMEM, 15 mM HEPES, 14 mM $NaHCO_3$, 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin, 2% (v/v) non-essential amino acids, and 1% (v/v) L-glutamine. DT40KO and DT40-IP_3R1 cells (gift of Colin Taylor) were cultured in RPMI 1640 medium, 15 mM HEPES, 14 mM $NaHCO_3$, 10% (v/v) FBS, 1% (v/v) heat-inactivated chicken serum, 1% (v/v) penicillin-streptomycin, 2 mM glutamine, and 50 μM 2-mercaptoethanol.

Measurement of Intracellular pH—Cells attached to glass coverslips were loaded with 5 μM (HEK-PR1 cells) or 7.5 μM (OK cells) 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) for 30 min at 37 °C and 5% (v/v) CO_2. pH_i was measured using a microspectrofluorometric system (excitation, 490/440 nm; emission, 535 nm). pH_i calibration was performed using high K^+ nigericin solutions (37).

cAMP Accumulation—Cells were starved overnight in 0.2% (w/v) BSA in serum-free medium and labeled for 2 h with 0.75 μCi ml^{-1} [^{3}H]adenine. Cells were washed with phosphate-buffered saline and incubated for 30 min at 37 °C at the desired CO_2 concentration in 990 μl of pre-incubation media (DMEM/F12 1:1 or DMEM depending on cell type, 15 mM HEPES, 1% (v/v) penicillin-streptomycin, 1 mM 3-isobutyl-1-methylxanthine) pre-gassed with the appropriate CO_2 concentration and with the pH adjusted. Assays were initiated with 10 μl of agonist. After 10 min at 37 °C, medium was removed, and cells were lysed with 1 ml 5% (w/v) trichloroacetic acid containing 1 mM ATP and 1 mM cAMP (OK, HEK-PR1, UMR-106 cells). cAMP was quantified by twin column chromatography (38). DT40KO and DT40-IP_3R1 cell cAMP was assayed using the Biotrak cAMP enzyme immunoassay (GE Healthcare) according to the manufacturers instructions. Antagonists were added to the pre-incubation media.

In Vitro Adenylyl Cyclase Assay—Cell monolayers were washed with phosphate-buffered saline and suspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM $CaCl_2$) for 20 min. The cell suspension was pelleted, re-suspended in lysis buffer, and incubated for a further 20 min. The cell suspension was pelleted and resuspended in 20 mM Tris-HCl, pH 7.5, 5 mM NaCl, 1 mM DTT, 1 mM 3-isobutyl-1-methylxanthine, 20% (v/v) glycerol, and homogenized through a 21-gauge needle. Adenylyl cyclase assays were performed at 37 °C in a final volume of 100 μl and contained 100 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 5 units of creatine phosphokinase, 5 μM creatine phosphate, and 1 mM [α^{32}P]ATP (25 kBq). Reactions were stopped by the addition of 150 μl of 50 mM Tris-HCl, pH 7.5, 5% (w/v) SDS. A further 650 μl of H_2O and 100 μl of 1 mM ATP, 1 mM [2,8-^{3}H]cAMP (150 Bq) were added prior to separation of product [α^{32}P]cAMP by the twin column method (38).

Measurement of NHE3 Activity—NHE3 activity was monitored by measuring pH_i recovery after a NH_4Cl pulse using BCECF-AM. OK cells were grown to 100% confluence on glass coverslips and starved overnight in 0.2% (v/v) BSA in serum-free media. 3-min NH_4Cl pulses (110 mM NaCl, 25 mM glucose, 20 mM NH_4Cl, 20 mM HEPES, 14 mM $NaHCO_3$, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.4) were followed by at least 5 min of perfusion in the same solution with NaCl replacing NH_4Cl.

Ca^{2+} Imaging—Cells were loaded with 10 μM of the Ca^{2+}-sensitive fluorescent dye Fura 2-AM in serum-free media for 30 min at 37 °C in 5% (v/v) CO_2 in air. Cells were washed and resuspended in Krebs-Ringer-HEPES solution (130 mM NaCl, 25 mM glucose, 20 mM HEPES, 14 mM $NaHCO_3$, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.4) for 30 min at 37 °C in 5% (v/v)

CO_2 in air. $CaCl_2$ was omitted when examining the effect of extracellular Ca^{2+}. Cells were transferred to fresh Krebs-Ringer-HEPES pre-gassed with the appropriate CO_2 concentration and the pH adjusted. Fura 2 emission was measured using a spectrofluorometer with simultaneous excitation at 340 and 380 nm and emission at 510 nm.

Statistical Analysis—Error bars represent the S.E. Statistical significance was determined by using Student's *t* test between indicated groups, unless otherwise indicated, and a 95% confidence interval was taken as $p < 0.05$.

RESULTS

The study of the effects of molecular CO_2 *in vivo* are confused by delineating CO_2 effects from those due to the associated acidosis and in differentiating between CO_2 effects on the tissue of interest from those secondary to changes in the endocrine and autonomic nervous systems. As elevated CO_2 influences renal processes regulated by cAMP (39), we studied a renal proximal tubule-derived cell line (OK cells (40)) as a model to investigate the impact of CO_2 upon cAMP signaling. A previous study had revealed that elevated (10%) CO_2 had no apparent influence on cAMP accumulation but a drop in cAMP-response element-binding protein phosphorylation suggested that elevated CO_2 might be inhibitory for cAMP signaling (12). Methodology was therefore developed on the basis of this study to investigate the influence of elevated CO_2 on cAMP signaling.

PTH couples to the cAMP generating enzyme AC through its cognate receptor and the G protein subunit, $G\alpha_s$. cAMP accumulation in OK cells was reduced at 10% compared with 5% CO_2 at a PTH concentration (5 nM) of similar magnitude to that used for previous analysis of the influence of CO_2 on OK cell physiology (Fig. 1*A*) (41). Batch to batch variation is known to influence the sensitivity of OK cells to PTH (42), but the response to CO_2 was independent of cell batch or passage number. The reduction in cAMP was independent of extracellular pH, as reduction of the medium pH from 7.5 to 7.0 did not affect the response (Fig. 1*B*). The EC_{50} for the response was unchanged as medium pH was dropped from 7.5 to 7.0 and cAMP accumulation actually increased (supplemental Fig. S1). The drop in cAMP in response to elevated CO_2 is therefore not explained by any potential acidification of medium pH on the assay. We measured final assay pH to assess whether changes in pH_e at elevated CO_2 might still influence the observed response through an effect on the potency and efficacy of PTH stimulation of AC. Final assay pH_e in assays performed at a starting pH_e of 7.5 at 5% CO_2 was 7.5 ± 0.1 (mean ± S.D.) and at 10% CO_2 was 7.4 ± 0.1 (mean ± S.D.). The observed drop in cAMP accumulation, caused by elevated CO_2, was therefore not unduly affected by any influence of pH_e on signaling. cAMP levels were depressed by elevated CO_2 when AC was directly activated by 10 μM forskolin (FSK), indicating that the effect of CO_2 is independent of the mechanism used to stimulate AC (Fig. 1*C*). The FSK response was insensitive to a drop in medium pH from 7.5 to 7.0 (supplemental Fig. S2) indicating, together with the relative stability of assay pH at elevated CO_2, that the response to CO_2 is not confused by any undue influence on pH_e. To differentiate between effects of molecular CO_2 and effects due to intracellular pH (pH_i), we examined the transient intracellular

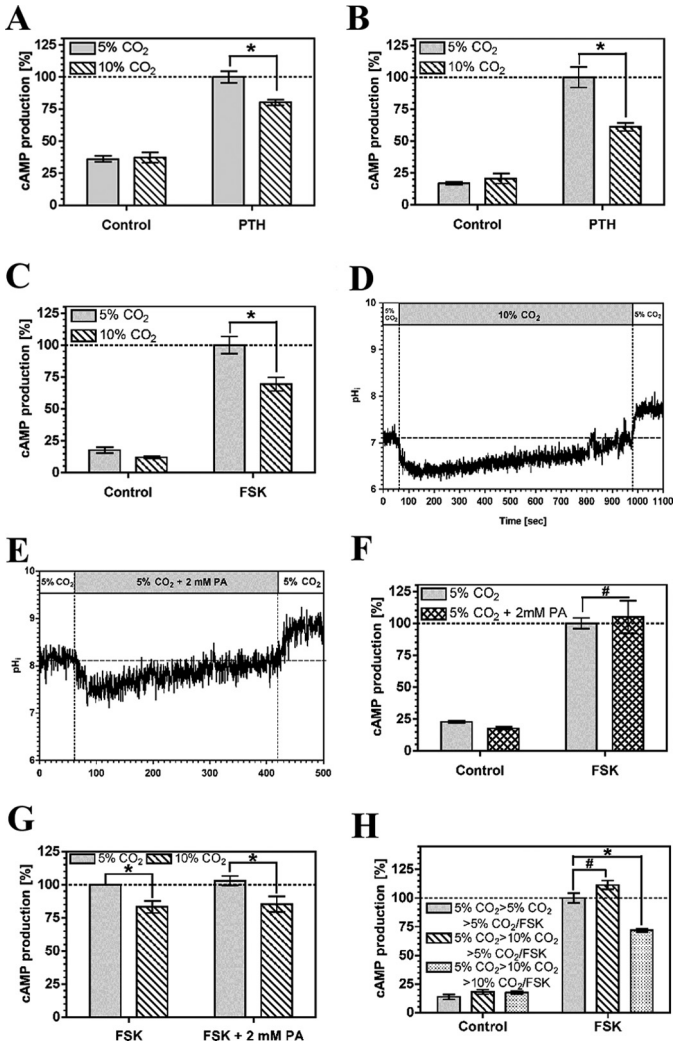
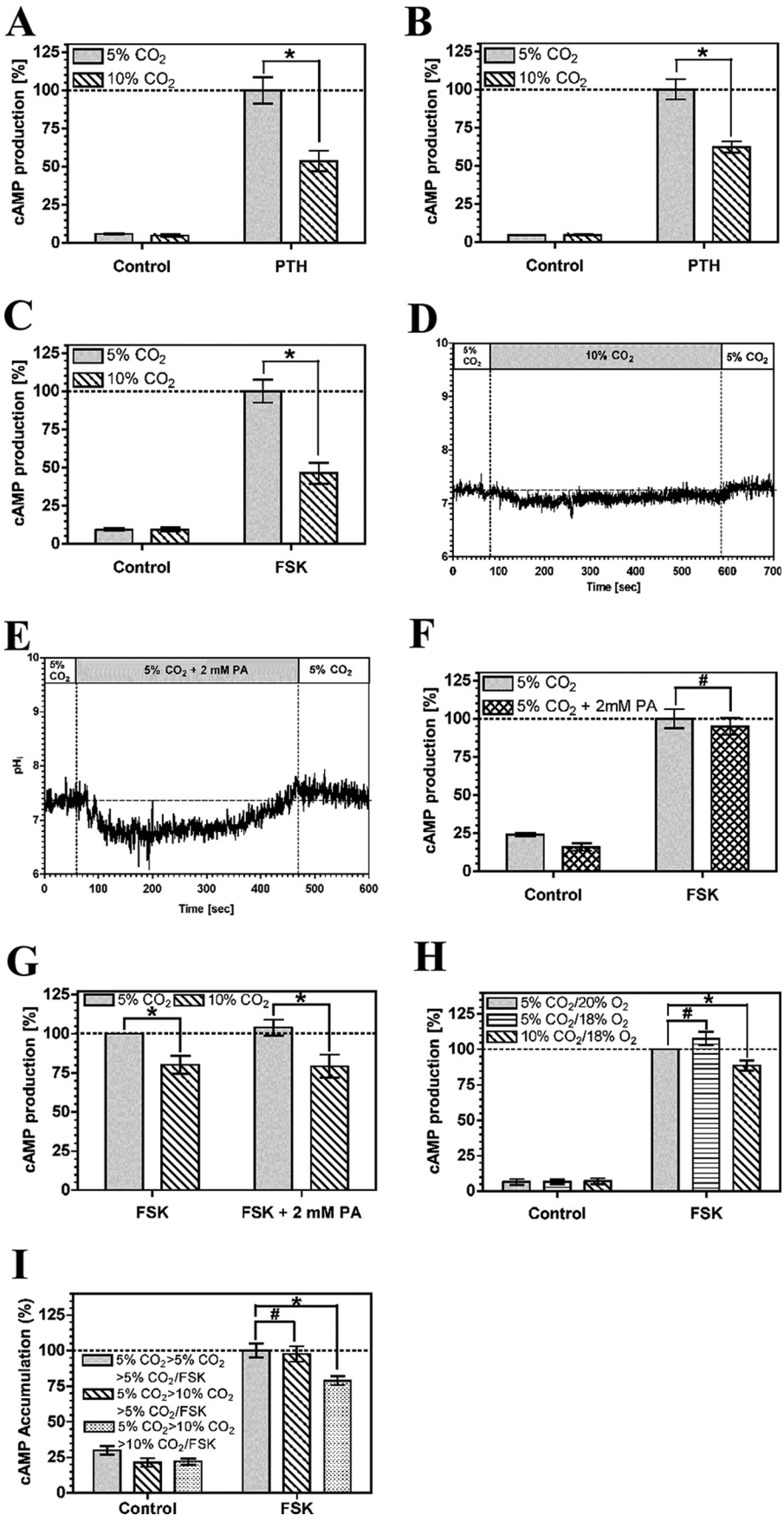


FIGURE 1. **Elevated CO_2 reduces cAMP accumulation in OK cells.** *A*, 5 nM PTH stimulated cAMP accumulation at 5% (v/v) CO_2 or 10% (v/v) CO_2 at pH 7.5. *B*, 5 nM PTH stimulated cAMP accumulation at 5% (v/v) CO_2 or 10% (v/v) CO_2 at pH 7.0. *C*, 10 μM FSK stimulated cAMP accumulation at 5% (v/v) CO_2 or 10% (v/v) CO_2 at pH 7.0. *D*, pH_i in OK cells in response to elevated CO_2. *E*, pH_i in OK cells in response to 2 mM propionic acid (*PA*). *F*, 10 μM FSK stimulated cAMP accumulation at 5% (v/v) CO_2 with or without 2 mM propionic acid at pH 7.5. *G*, 10 μM FSK stimulated cAMP accumulation at 5% (v/v) CO_2 or 10% (v/v) CO_2 with 2 mM propionic acid at pH 7.5. *H*, OK cells at 5% (v/v) CO_2 were transferred to 5% (v/v) CO_2 or 10% (v/v) CO_2 for 30 min before assay at 5% (v/v) CO_2 or 10% (v/v) CO_2 with 10 μM FSK (pH 7.5). cAMP accumulation in each graph is normalized to the value in the presence of agonist at 5% (v/v) CO_2 ($n > 3$; *, $p < 0.05$; #, not significant).

acidification caused by CO_2 ($\Delta pH_i = -0.72 \pm 0.17$; Fig. 1*D*). We approximated the extent of intracellular acidification with media containing 2 mM propionic acid ($\Delta pH_i = -0.60 \pm 0.35$; Fig. 1*E*). Transient intracellular acidification by propionic acid had no influence on cAMP indicating that the effect of CO_2 on cAMP is not mediated through pH_i (Fig. 1*F*). Furthermore, propionic acid did not influence the response of cAMP to CO_2, indicating that propionic acid does not influence the cAMP pathway such that it cannot respond to inhibitory signals (Fig. 1*G*). The effect of CO_2 on cAMP levels was fully reversible. Cells grown at 5% CO_2 and then exposed to 10% CO_2 prior to assay at 5% CO_2 with FSK demonstrated cAMP accumulation indistinguishable from cells maintained and assayed at 5% CO_2 (Fig. 1*H*). We examined whether the effect of CO_2 on AC activ-


A
cAMP production [%]
5% CO2
10% CO2
Control
PTH
B
cAMP production [%]
5% CO2
10% CO2
Control
PTH
C
cAMP production [%]
5% CO2
10% CO2
Control
FSK
D
5% CO2
10% CO2
5% CO2
Time [sec]
E
5% CO2
5% CO2 + 2 mM PA
5% CO2
pHi
Time [sec]
F
cAMP production [%]
5% CO2
5% CO2 + 2mM PA
Control
FSK
G
cAMP production [%]
5% CO2
10% CO2
FSK
FSK + 2 mM PA
H
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5% CO2/20% O2
5% CO2/18% O2
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5% CO2>10% CO2 >5% CO2/FSK
5% CO2>10% CO2 >10% CO2/FSK
Control
FSK

ity was due to protein degradation. The *in vitro* AC activity of OK cell crude membrane preparations exposed to 5 or 10% CO_2 was similar (the specific activity at 10% CO_2 was 115 ± 8% (S.E., $n = 6$) that at 5% CO_2), indicating similar protein levels and consistent with the reversibility of the response to CO_2.

To determine whether the CO_2 effect was cell context-specific, we examined the response of HEK 293 cells stably transfected with the human type 1 PTH receptor (HEK-PR1 cells) (43). cAMP accumulation stimulated by 5 nM PTH was reduced at elevated CO_2 independent of extracellular pH (Fig. 2, *A* and *B*, supplemental Fig. S1) and any specific pathway used to stimulate AC (10 μM FSK; Fig. 2*C* and supplemental Fig. S2). 2 mM propionic acid gave a drop in pH_i ($\Delta pH_i = -0.61 \pm 0.32$; Fig. 2*E*) greater than that for elevated CO_2 ($\Delta pH_i = -0.21 \pm 0.14$; Fig. 2*D*) but had no influence on cAMP accumulation (Fig. 2*F*). Similar to OK cells, propionic acid did not influence the response of cAMP to CO_2 (Fig. 2*G*). As the experimental process of elevating CO_2 in air causes a small hypoxic effect (from 19.9% to 18.9% O_2 (v/v)) we examined whether a shift from 20% to 18% O_2 at constant CO_2 influenced cAMP levels as an additional control. The mild hypoxia had no influence on cAMP indicating that the effect is mediated through CO_2 (Fig. 2*H*). Similar to OK cells, the *in vitro* AC activity of HEK-PR1 crude membrane preparations exposed to 5 or 10% CO_2 was similar (the specific activity at 10% CO_2 was 99 ± 5% (S.E., $n = 5$) that at 5% CO_2) indicating similar protein levels and cells grown at 5% CO_2 and then exposed to 10% CO_2 prior to assay at 5% CO_2 with FSK demonstrated cAMP accumulation indistinguishable from cells maintained and assayed at 5% CO_2 (Fig. 2*I*). To confirm that the effect of elevated CO_2 on cAMP is a broadly applicable phenomenon we also demonstrated that the PTH-responsive rat osteosarcoma cell line, UMR-106 (44), showed an identical response to CO_2 (supplemental Fig. S3).

The activity of the nine identified mammalian G protein-responsive AC isoforms can be modulated by a number of other signaling processes, and we investigated whether any of these pathways was responsible for the reduction in cAMP accumulation in response to CO_2. We used antagonists whose broad target range enabled us to simultaneously inhibit multiple cAMP interacting signaling pathways (Fig. 3*A*). The effect of CO_2 on cAMP accumulation in HEK-PR1 cells did not require the activity of cAMP phosphodiesterase (1 mM 3-isobutyl-1-methylxanthine), soluble adenylyl cyclase (10 μM KH7), cAMP-dependent protein kinase (PKA) (10 μM H-89), calcium-calmodulin-dependent protein kinase II (100 nM autocamtide II), or protein kinase C (1 μM staurosporine/1 mM Gö 6983). The lack of an effect of CO_2 with the AC inhibitor SQ 22,536 (200 μM) demonstrated the requirement for a G protein-responsive AC (45) as opposed to soluble AC, which is unresponsive to SQ 22,536 (46, 47). Carbonic anhydrase inhibition (100 μM acetazolamide) had no effect, indicating no requirement for conversion of CO_2 to HCO_3^- (14). 1 mM extracellular ethylene glycol tetraacetic acid (EGTA) had no effect on the CO_2 response, whereas it was ablated by the acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM; 1 mM), indicating a requirement for intracellular but not extracellular Ca^{2+}. The influence of BAPTA-AM on the CO_2 response was confirmed in OK cells, demonstrating a likely common mechanism of action (Fig. 3*B*). We used further inhibitors to investigate the source of the intracellular Ca^{2+}. The CO_2 effect was insensitive to 100 μM nifedipine (L- and T-type voltage-dependent Ca^{2+} channel blocker) in both OK and HEK-PR1 cells and 5 μM rotenone (a mitochondrial inhibitor) in HEK-PR1 cells (Fig. 4, *A* and *B*). Rotenone ablated the response of cAMP to CO_2 in OK cells; however, we noted sig-

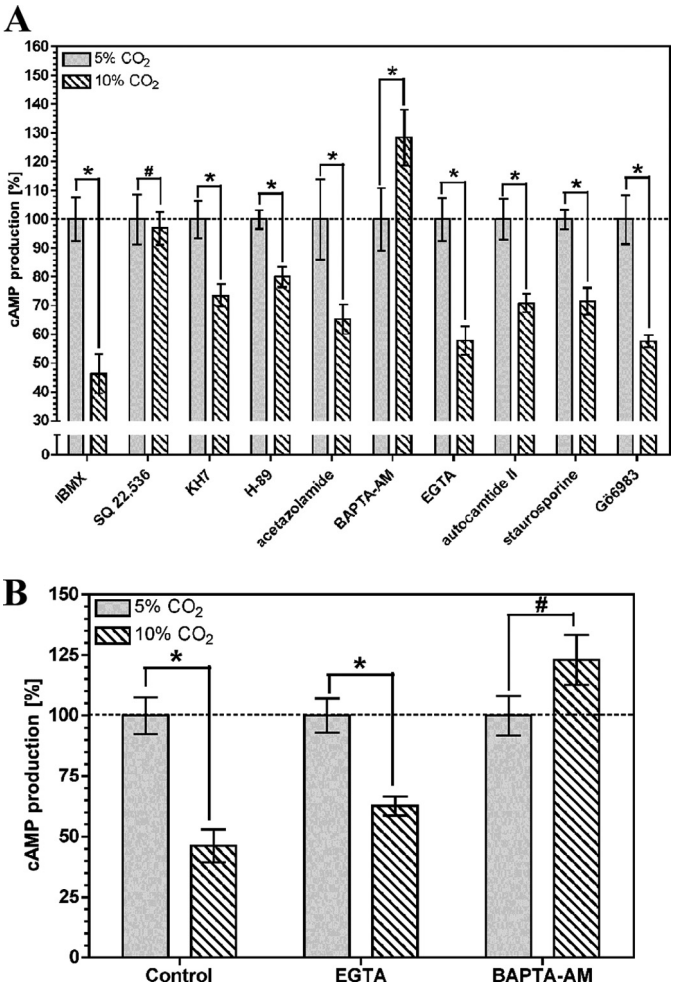


FIGURE 3. **Intracellular Ca^{2+} is required for CO_2 to reduce cellular cAMP.** cAMP accumulation in HEK-PR1 (*A*) or OK cells (*B*) exposed to 5% (v/v) CO_2 or 10% (v/v) CO_2 at pH 7.5 in the presence of 10 μM FSK and various antagonists. cAMP accumulation for each antagonist is normalized to the value at 5% (v/v) CO_2 ($n > 4$; *, $p < 0.05$; #, not significant). *IBMX*, 3-isobutyl-1-methylxanthine.

FIGURE 2. **Elevated CO_2 reduces cAMP accumulation in HEK-PR1 cells.** *A*, 5 nM PTH stimulated cAMP accumulation at 5% (v/v) CO_2 or 10% (v/v) CO_2 at pH 7.5. *B*, 5 nM PTH stimulated cAMP accumulation at 5% (v/v) CO_2 or 10% (v/v) CO_2 at pH 7.0. *C*, 10 μM FSK stimulated cAMP accumulation at 5% (v/v) CO_2 or 10% (v/v) CO_2 at pH 7.0. *D*, pH_i in HEK-PR1 cells in response to elevated CO_2. *E*, pH_i in HEK-PR1 cells in response to 2 mM propionic acid. *F*, 10 μM FSK stimulated cAMP accumulation at 5% (v/v) CO_2 with or without 2 mM propionic acid at pH 7.5. *G*, 10 μM FSK stimulated cAMP accumulation at 5% (v/v) CO_2 or 10% (v/v) CO_2 with 2 mM propionic acid (*PA*) at pH 7.5. *H*, 10 μM FSK stimulated cAMP accumulation at 5% (v/v) CO_2/20% (v/v) O_2, 5% (v/v) CO_2/18% (v/v) O_2, or 10% (v/v) CO_2/18% (v/v) O_2 at pH 7.5. *I*, HEK-PR1 cells at 5% (v/v) CO_2 were transferred to 5% (v/v) CO_2 or 10% (v/v) CO_2 for 30 min before assay at 5% (v/v) CO_2 or 10% (v/v) CO_2 with 10 μM FSK (pH 7.5). cAMP accumulation in each graph is normalized to the value in the presence of agonist at 5% (v/v) CO_2 ($n > 3$; * $p < 0.05$; #, not significant).

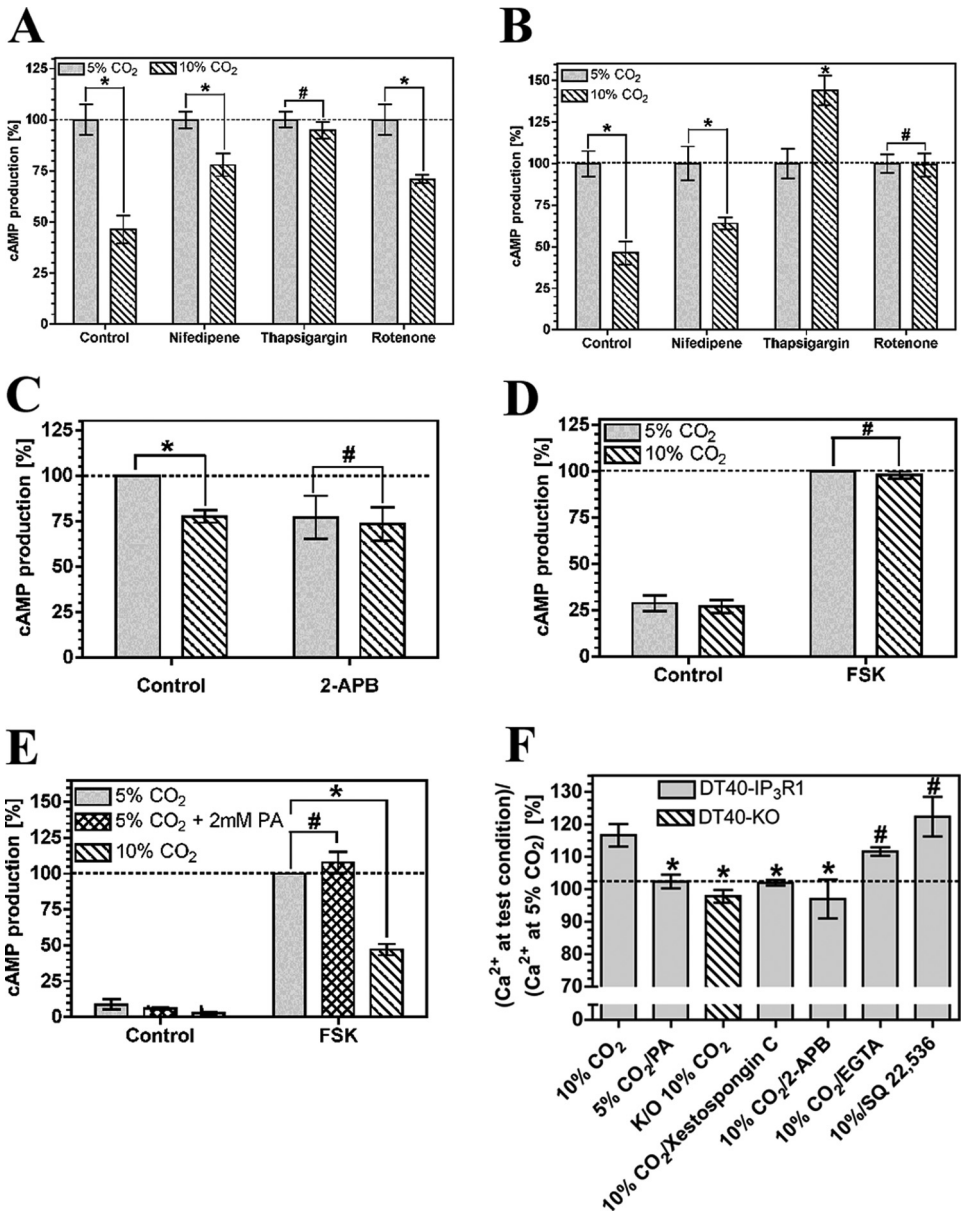
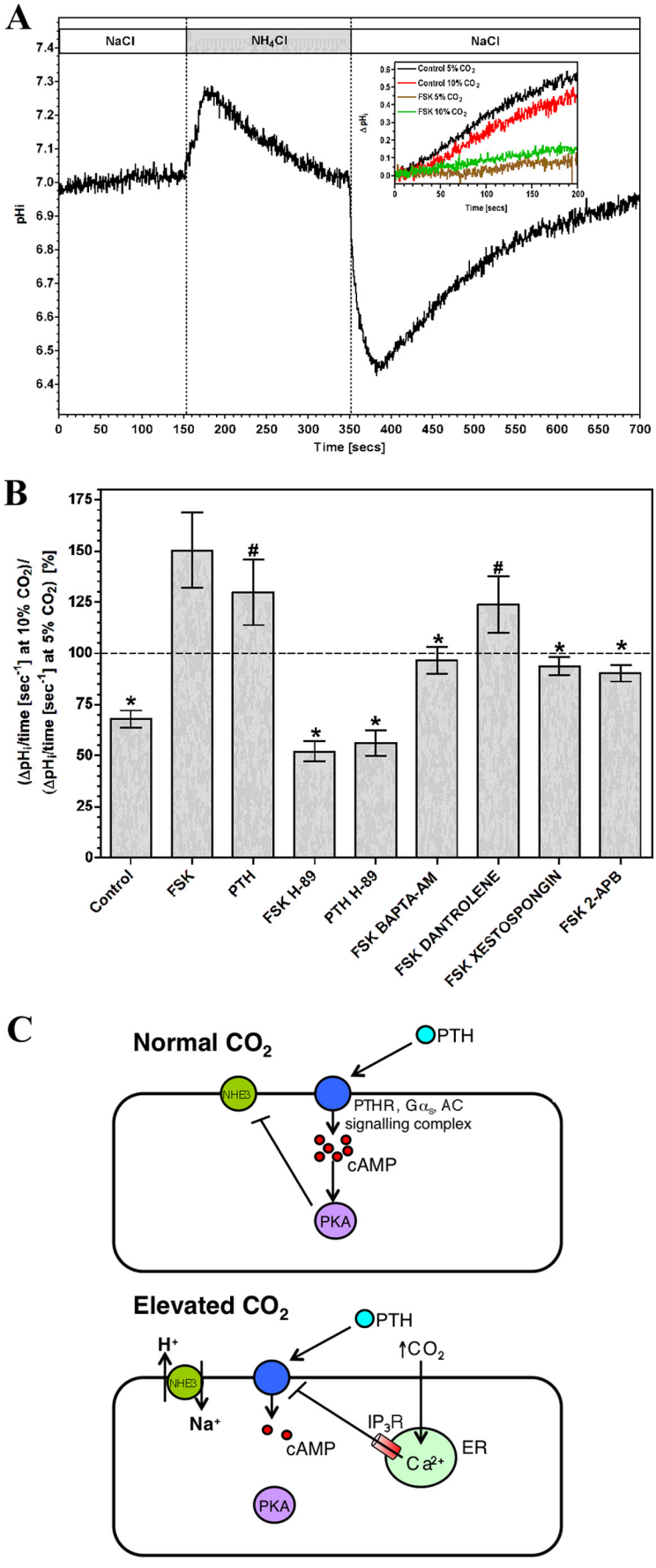


FIGURE 4. **Ca^{2+} release via IP_3R is required for CO_2 to reduce cellular cAMP.** *A–C*, cAMP accumulation in HEK-PR1 (*A* and *C*) or OK (*B*) cells exposed to 5% (v/v) CO_2 or 10% (v/v) CO_2 at pH 7.5 in the presence of 10 μM FSK and various antagonists. cAMP accumulation for each antagonist is normalized to the value at 5% (v/v) CO_2 ($n > 4$; *, $p < 0.05$; #, not significant). *D* and *E*, 10 μM FSK stimulated cAMP accumulation in DT40KO (*D*), and DT40-IP_3R1 cells (*E*) exposed to 5% (v/v) CO_2, 10% (v/v) CO_2, or 5% (v/v) CO_2 with 2 mM propionic acid (*PA*) at pH 7.5. cAMP accumulation in each graph is normalized to the value at 5% (v/v) CO_2 ($n > 3$; *, $p < 0.05$; #, not significant). *F*, ratio of cytosolic Ca^{2+} at a test condition *versus* 5% (v/v) CO_2 at pH 7.5 in DT40-IP_3R1 or DT40-KO cells in the presence of various antagonists ($n > 6$; *, $p < 0.05$; #, not significantly <10% (v/v) CO_2 by one-way analysis of variance with post-hoc one-sided Dunnett test).

nificant toxicity and cell death in response to rotenone in this cell line, indicating that this might be a nonspecific effect. The cAMP response was ablated by 10 μM thapsigargin (endoplasmic reticulum Ca^{2+}-ATPase inhibitor) in both HEK-PR1 and OK cells (Fig. 4, *A* and *B*). This indicated a likely role for CO_2 mediated Ca^{2+} release from a thapsigargin-sensitive store, most likely via the IP_3 receptor and the endoplasmic reticulum. Further evidence for this was obtained in HEK-PR1 cells using the IP_3R inhibitor 100 μM 2-APB (Fig. 4*C*). To eliminate the possibility of off target effects with thapsigargin and 2-APB, particularly as significant variability was observed with the latter due to toxicity, we investigated more specific evidence for the involvement of the IP_3 receptor in Ca^{2+} release. We examined the effect of elevated CO_2 on the DT40KO cell line (48). DT40KO cells are a chicken B lymphocyte-derived cell line genetically ablated for type 1, 2, and 3 IP_3 receptors and are a null background for IP_3 receptor studies. We examined cAMP accumulation in response to elevated CO_2 in DT40KO cells compared with DT40-IP_3R1 cells that have had the rat IP_3 type 1 receptor introduced (49). Elevated CO_2 did not blunt cAMP accumulation in DT40KO cells (Fig. 4*D*) but did in DT40-IP_3R1 cells (Fig. 4*E*), proving that the type 1 IP_3 receptor is required for the response to CO_2. Intracellular acidification through 2 mM propionic acid had no influence on cellular cAMP in DT40-IP_3R1 cells as observed for both OK and HEK-PR1 cells.


A
NaCl
NH4Cl
NaCl
pHi
Time [secs]
Control 5% CO2
Control 10% CO2
FSK 5% CO2
FSK 10% CO2
ΔpHi
B
(ΔpHi/time [sec-1] at 10% CO2)/
(ΔpHi/time [sec-1] at 5% CO2) [%]
Control
FSK
PTH
FSK H-89
PTH H-89
FSK BAPTA-AM
FSK DANTROLENE
FSK XESTOSPONGIN
FSK 2-APB
C
Normal CO2
PTH
NHE3
PTHR, Gαs, AC
signalling complex
cAMP
PKA
Elevated CO2
PTH
H+
↑CO2
NHE3
Na+
cAMP
IP3R
Ca2+
ER
PKA

As the CO_2/cAMP effect is sensitive to intracellular Ca^{2+} chelation and shows a requirement for the IP_3 receptor, we investigated whether elevated CO_2 altered steady state cytoplasmic [Ca^{2+}] in both DT40KO cells and DT40-IP_3R1 cells. Cytoplasmic [Ca^{2+}] was elevated at 10% compared with 5% CO_2 in DT40-IP_3R1 but not DT40KO cells (Fig. 4*F*). Nonspecific intracellular acidification through propionic acid had no influence on cytoplasmic [Ca^{2+}]. Elevated CO_2 therefore mediates Ca^{2+} release from the endoplasmic reticulum via the IP_3 receptor. To provide an independent validation for the role of the IP_3 receptor in the DT40 cell response to CO_2, we treated DT40-IP_3R cells with either 1 mM EGTA, 100 μM 2-APB, or 500 nM xestospongin C (Fig. 4*F*). Cytoplasmic [Ca^{2+}] was elevated at 10% compared with 5% CO_2 in DT40-IP_3R1 cells in the presence of EGTA, consistent with the absence of an effect of EGTA on CO_2 modulation of cAMP. The Ca^{2+} release in response to CO_2 was ablated by the IP_3 receptor antagonists xestospongin C and 2-APB. This result supports the interpretation that these inhibitors are blocking IP_3 receptor signaling in HEK-PR1 and OK cells (Figs. 4*C* and 5*B*). Inclusion of the AC inhibitor SQ 22,536 (200 μM) had no influence on CO_2-mediated Ca^{2+} release. These data confirm that an increase in cytosolic Ca^{2+} is a prerequisite for the effect of CO_2 on cAMP (Fig. 4, *D* and *E*) and that cAMP lies downstream of the increase in cytosolic Ca^{2+} (Fig. 4*F*).

We investigated the functional consequences of CO_2-mediated reductions in intracellular cAMP by assessing a cAMP-dependent physiological process in OK cells. Sodium-proton exchanger isoform 3 (NHE3) is an apical Na^+-H^+ antiporter of renal epithelial (and OK) cells with a crucial role in H^+, Na^+, and fluid homeostasis (50). NHE3 is inhibited by PKA phosphorylation at serine residues 552 and 605 (51), and we examined the effect of elevated CO_2 on cAMP-mediated suppression of NHE3 activity. OK cells exposed to NH_4Cl alkalize due to H^+ buffering by NH_3, but pH regulatory mechanisms returns pH_i to normal (Fig. 5*A*). On exchange of NH_4Cl for NaCl, pH_i drops as the accumulated intracellular NH_4^+ releases H^+. The alkalization to restore pH_i is due to NHE3 (52), and we analyzed this phase of the response.

Comparison of control pH_i recoveries at 5 and 10% CO_2 demonstrated a cAMP-independent suppression of recovery at elevated CO_2 (Fig. 5, *A* and *B*; note the ratio of recovery at 10% compared with 5% $CO_2 < 1$). Inhibition of NHE3-mediated pH_i recovery by FSK or PTH was greater in 5% compared with 10% CO_2 consistent with the effect of CO_2 on cAMP levels (note the ratio of recovery at 10% CO_2 compared with 5% $CO_2 > 1$). The effect of CO_2 on cAMP inhibition of pH_i recovery was reduced by H-89 and BAPTA-AM, demonstrating a requirement for both PKA and intracellular Ca^{2+}. 10 μM dantrolene (a ryanodine receptor antagonist) had no influence on CO_2 suppression of cAMP signaling. The IP_3 receptor antagonists xestospongin C (500 nM) and 2-APB (100 μM) eliminated the effect of CO_2 on cAMP-dependent NHE3 inhibition. CO_2 therefore suppresses the activity of the cAMP signaling pathway through Ca^{2+} release via the IP_3 receptor with functional consequences for cAMP-dependent cellular processes (Fig. 5*C*).

DISCUSSION

In this work, we make two original claims. The first is that molecular CO_2 reduces levels of cellular cAMP when the G-protein responsive cAMP signaling pathway is activated and this has functional consequences for downstream processes. This is in direct contrast to the current paradigm where cyclic nucleotide levels, where they are observed to respond to CO_2, increase. The second is that molecular CO_2 increases steady state cytoplasmic Ca^{2+} concentrations dependent on the IP_3 receptor. The effect of CO_2 on cAMP is a consequence of this altered Ca^{2+}. These findings significantly advance our understanding of the effects of CO_2 on the cell.

CO_2 toxicity is not straightforward to study due to the effects of the associated acidosis and secondary effects on the endocrine and autonomic nervous systems. We circumvented these problems through the use of cultured cells to demonstrate that elevated CO_2 blunts cellular cAMP production independent of pH. The use of sub-maximally activating concentrations of cAMP stimulating agonists enabled us to detect either activation or down-regulation of the cAMP signaling pathway under any given experimental condition. Submaximal 5 nM PTH gave only a very small increase in cAMP in experiments to examine the influence of pH_e in OK cells (supplemental Fig. S1). As the assay procedure used has minimal effects on pH_e, this issue with this batch of cells does not affect the main findings. Cell batches used to examine the influence of CO_2 gave robust responses at submaximal 5 nM PTH (Figs. 1 and 5). In addition to a failure of intracellular acidification to modulate cAMP, two further lines of evidence make it unlikely that the effects of CO_2 are mediated by pH_i. First, pH_i is allowed to normalize after CO_2 elevation requiring a hypothesized acid signal to persist long after pH homeostasis. Second, FSK (activating all AC isoforms) and PTH (coupling to AC6 in HEKPR1 cells (53)) give identical responses to CO_2 arguing against a localized acid signal communicating with a distinct signaling enzyme. Extracellular pH is also unlikely to be responsible as medium acidification (as might be proposed to occur mid-assay were buffering insufficient) does not alter the EC_{50} for PTH and actually gives an increase in cAMP. Medium acidification would therefore cause an underestimation of the decrease in cAMP. An increase in pCO_2 causes a small decrease in pO_2, but the decrease in pO_2 did not explain the effects of pCO_2 on cAMP. We conclude, therefore, that the effect of hypercapnia is mediated by molecular CO_2 and not pH or any other variable.

The influence of CO_2 on cAMP signaling might be due to a direct interaction with AC or with an alternative signaling pathway, which impacts on cAMP levels. Pharmacological inhibi-

FIGURE 5. **Elevated CO_2 blunts cAMP inhibition of NHE3 in OK cells.** *A*, monitoring of pH_i in OK cells in response to NH_4Cl. The *inset* shows a sample experiment monitoring post-alkalization pH_i recovery analyzed to generate the graph below. *B*, ratio of slope of pH_i recovery at 10% (v/v) CO_2 *versus* 5% (v/v) CO_2 after shift from NH_4Cl to NaCl in the presence of 10 μM FSK or 5 nM PTH and various inhibitors ($n > 3$; *, $p < 0.05$; #, not significant compared with FSK by one-way analysis of variance with post-hoc Bonferroni test). *C*, model for the impact of CO_2 on PTH signaling at normal and elevated CO_2. *ER*, endoplasmic reticulum.

tors offered the best initial route to investigate CO_2 responsive pathways that influence cAMP as the experiment could exploit the broad spectrum of targets antagonised under moderate concentrations. This method identified a role for intracellular Ca^{2+} in the response to CO_2. The Ca^{2+} was most likely derived from an intracellular store as external EGTA did not influence the response. Further support came from the use of inhibitors previously used to study the role of Ca^{2+} in cellular responses to CO_2 (27). Rotenone antagonized the cellular response to CO_2 in OK cells, but this is most likely due to its observed toxicity, particularly as the effect was not reproduced in HEK-PR1 cells. Thapsigargin ablated the cAMP response to CO_2 in both OK and HEK-PR1 cells. The use of 2-APB, which inhibits both the IP_3 receptor and transient receptor potential channels, supported this finding. The lack of a role for extracellular Ca^{2+} eliminated the transient receptor potential channels and supported a role for Ca^{2+} release from the endoplasmic reticulum, in particular via the IP_3 receptor. A role for the IP_3 receptor was proven through the use of a cell line genetically ablated for all three IP_3 receptor isoforms. The most likely interpretation of the pharmacological analysis in HEK-PR1 and OK cells is therefore supported unambiguously through genetics in an independent cell line. It is formally possible that the underlying signaling response to CO_2 in HEK-PR1 and OK cells does not involve the IP_3 receptor. This possibility is unlikely, however, as the IP_3 receptor antagonists used in this study were validated by demonstrating that their effect on CO_2-mediated Ca^{2+} release in DT40-IP_3R1 cells was identical to the effect of the genetic ablation. A combination of the subtlety of the effects of CO_2 and the incomplete knockdown of the IP_3 receptor using siRNA (53) makes a genetic validation for the role of the IP_3 receptor in these cells currently unfeasible. Future experiments using knock-outs generated via zinc finger nucleases will permit a genetic approach to be adopted in these cells.

Despite this, a coherent picture does emerge and supports what is known of the toxicity of CO_2. A role for altered Ca^{2+} signaling in the response to CO_2 is consistent with generic toxicity as Ca^{2+} signaling is a core physiological process evident in all mammalian cell types and elevated cytosolic Ca^{2+} is associated with cell toxicity (54). The approximate 20% increase in steady state cytoplasmic Ca^{2+} and 10–50% drop in cellular cAMP are relatively subtle effects and are consistent with the reasonable tolerance of mammals to elevated CO_2 (55). A role for Ca^{2+} in CO_2 responses has been investigated previously. Vadász and co-workers (32) noted a transient increase in cytoplasmic Ca^{2+} in response to hypercapnia, whereas Bouyer and co-workers (27) identified a rise in intracellular Ca^{2+} proposed as originating from an unconventional source. Our work provides significant clarity in its use of genetics to identify the Ca^{2+} source, but we cannot eliminate the possibility of context-dependent Ca^{2+} sources. It will be instructive in the future to investigate whether the effects of Ca^{2+} on AC are direct (56) or occur through a Ca^{2+}-sensitive signaling pathway (57).

A crucial discovery is that elevated CO_2 suppresses a cAMP-dependent cellular process. The effect of CO_2 on a cAMP-responsive Na^+-H^+ antiporter is consistent with the observed biochemistry of the influence of CO_2 on cAMP. We propose, therefore, that altered cAMP and/or Ca^{2+} signaling can be investigated further as a key mechanism by which the toxic effects of CO_2 are manifested. For example, there is growing evidence that chronic daytime hypercapnia associated with obstructive sleep disorders predisposes individuals to cardiovascular disease (58). The central role of cAMP and Ca^{2+} in cardiac and circulatory physiology (57) suggests a key route to understanding this pathophysiology and possibilities for therapeutic intervention.

Ca^{2+} efflux through the IP_3 receptor is modulated both by signaling pathways that regulate phospholipase C and by the local cellular environment (59–61). A major challenge for the future will be to determine how CO_2 causes Ca^{2+} release through the IP_3 receptor and its direct cellular target. Identification of such a CO_2 target will further inform our understanding of the cell biology of CO_2, in particular other cell processes that are influenced by CO_2 to mediate its toxicity.

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