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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 $\mu$g/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-$IP_3$R1 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 $\mu$M 2-mercaptoethanol.

*Measurement of Intracellular pH*—Cells attached to glass coverslips were loaded with 5 $\mu$M (HEK-PR1 cells) or 7.5 $\mu$M (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 $\mu$Ci 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 $\mu$l 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 $\mu$l 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-$IP_3$R1 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 $\mu$l 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 $\mu$M creatine phosphate, and 1 mM [$\alpha^{32}$P]ATP (25 kBq). Reactions were stopped by the addition of 150 $\mu$l of 50 mM Tris-HCl, pH 7.5, 5% (w/v) SDS. A further 650 $\mu$l of $H_2O$ and 100 $\mu$l of 1 mM ATP, 1 mM [2,8-$^3$H]cAMP (150 Bq) were added prior to separation of product [$\alpha^{32}$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 $\mu$M 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

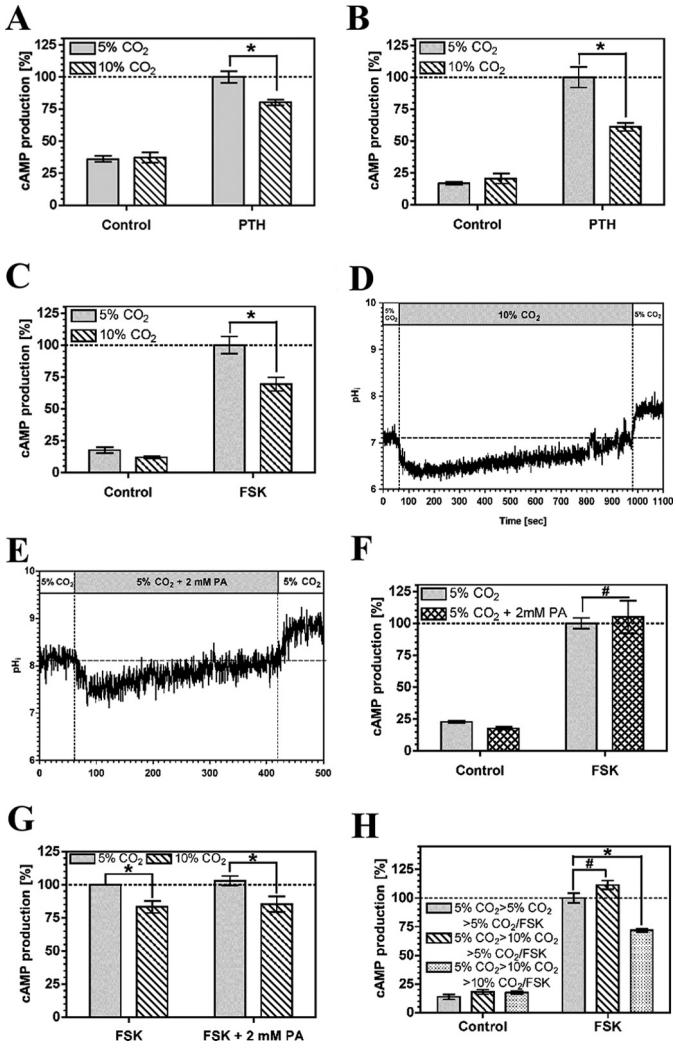
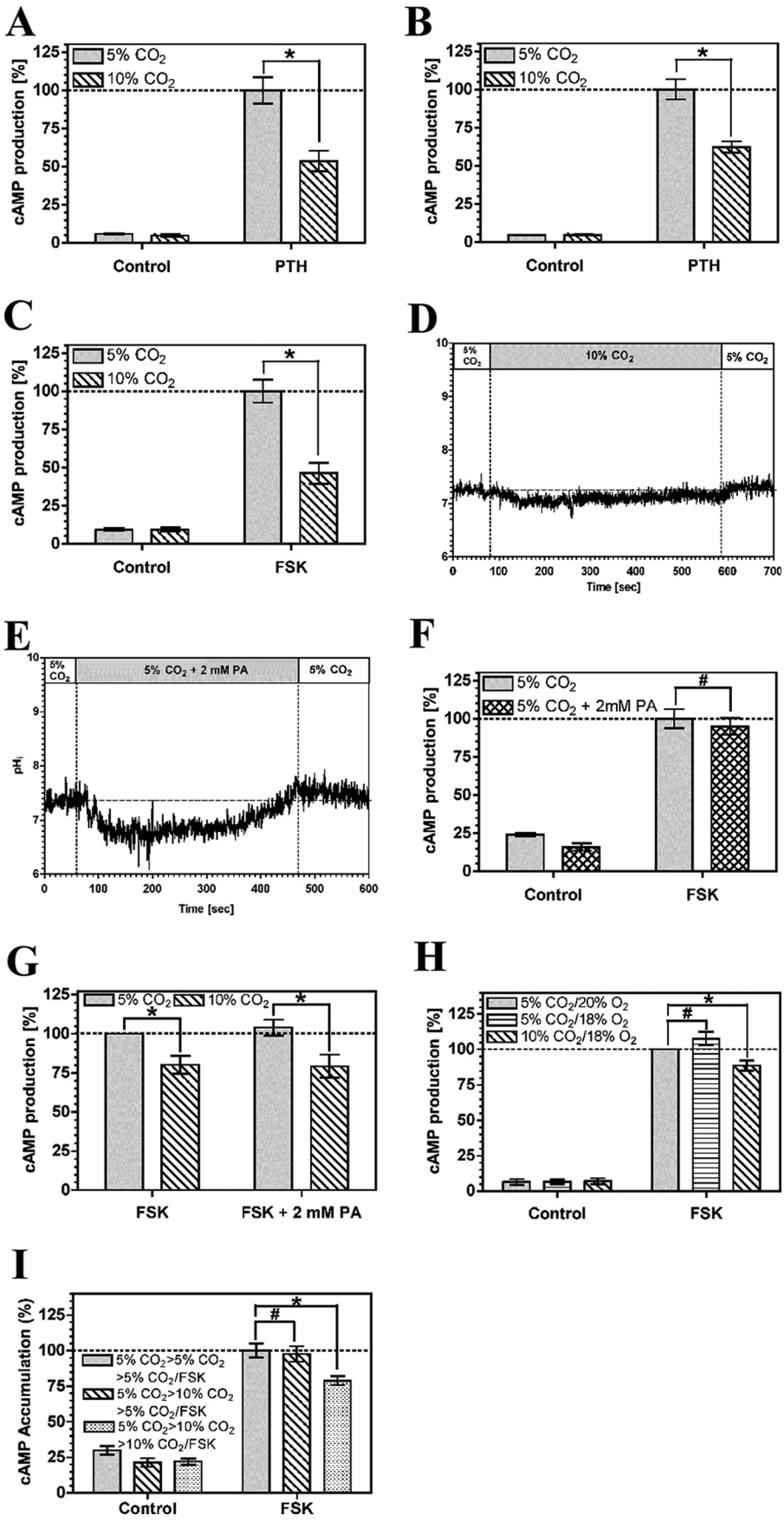



FIGURE 1. **Elevated $CO_2$ reduces cAMP accumulation in OK cells.** *A*, 5 nM PTH stimulated cAMP accumulation at 5% (v/v) $CO_2$ or 10% (v/v) $CO_2$ at pH 7.5. *B*, 5 nM PTH stimulated cAMP accumulation at 5% (v/v) $CO_2$ or 10% (v/v) $CO_2$ at pH 7.0. *C*, 10 μM FSK stimulated cAMP accumulation at 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-



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-

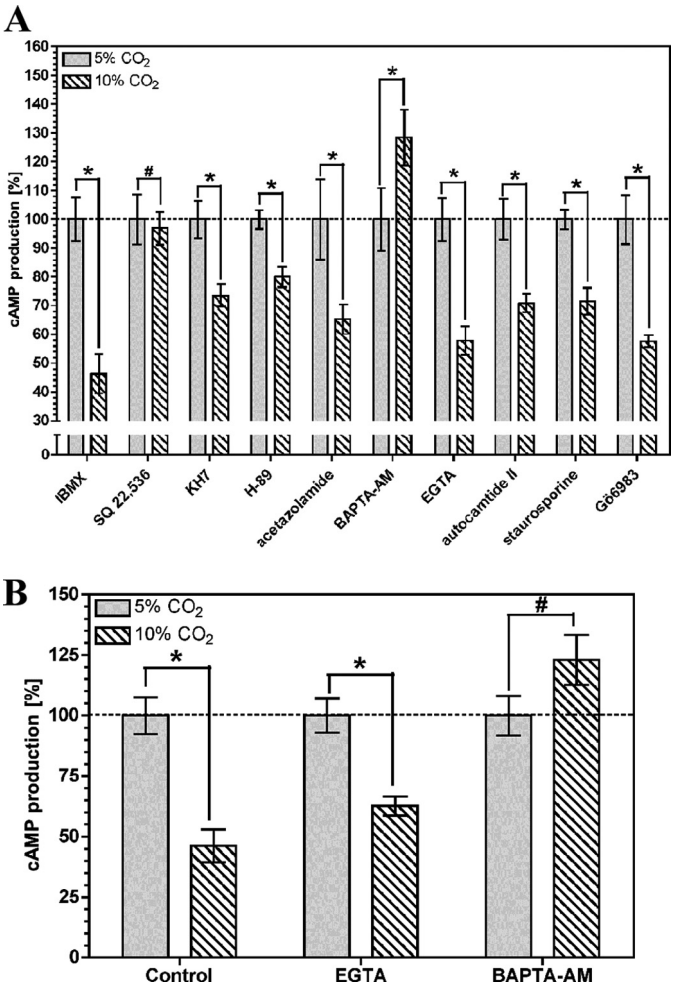



FIGURE 3. **Intracellular $Ca^{2+}$ is required for $CO_2$ to reduce cellular cAMP.** cAMP accumulation in HEK-PR1 (*A*) or OK cells (*B*) exposed to 5% (v/v) $CO_2$ or 10% (v/v) $CO_2$ at pH 7.5 in the presence of 10 μM FSK and various antagonists. cAMP accumulation for each antagonist is normalized 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).

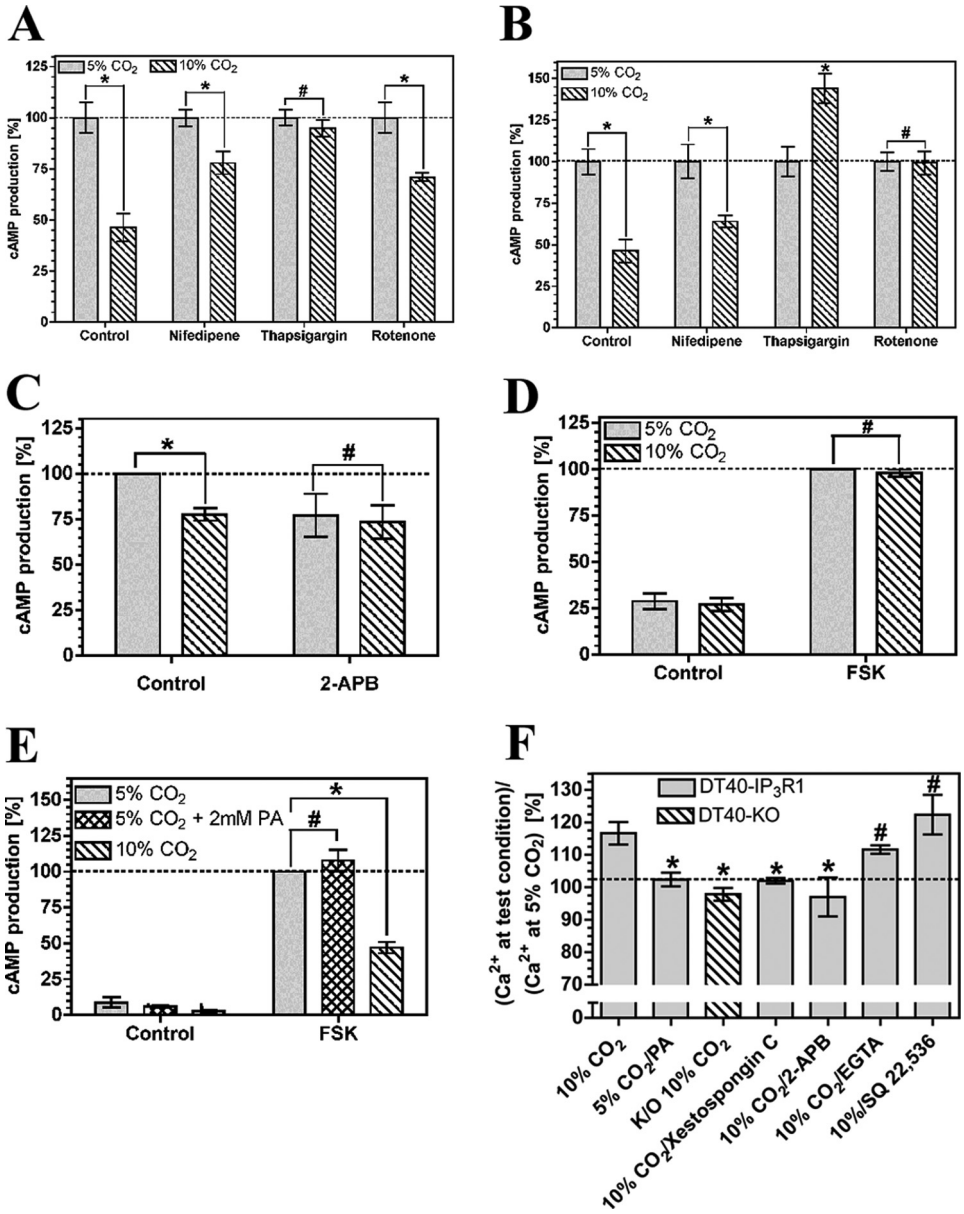
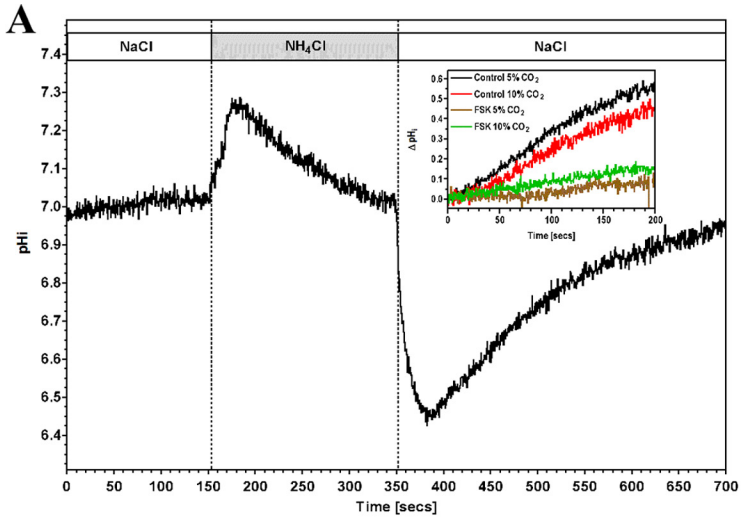
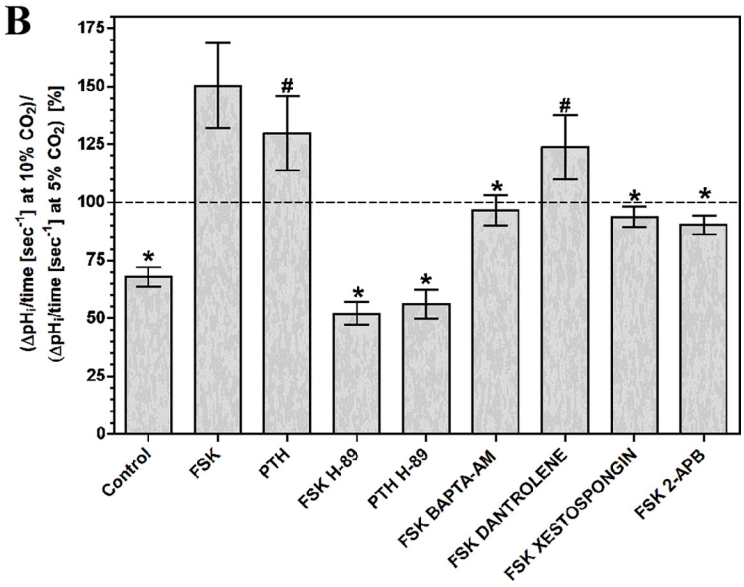
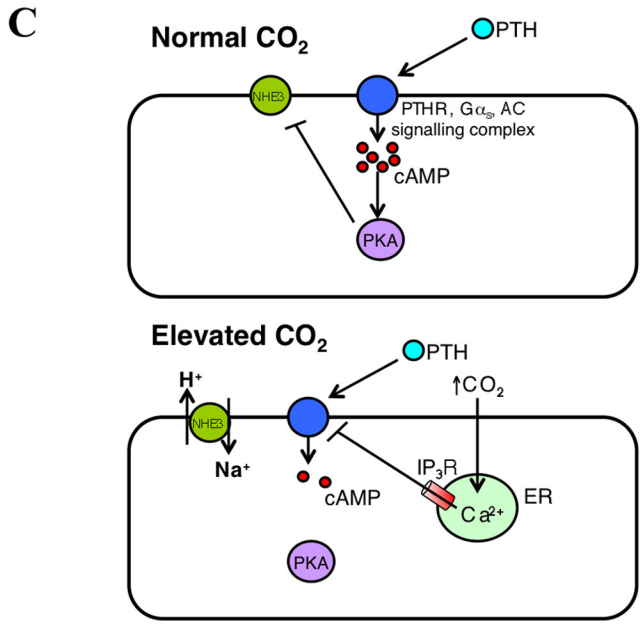



FIGURE 4. **$Ca^{2+}$ release via $IP_3R$ is required for $CO_2$ to reduce cellular cAMP.** *A–C*, cAMP accumulation in HEK-PR1 (*A* and *C*) or OK (*B*) cells exposed to 5% (v/v) $CO_2$ or 10% (v/v) $CO_2$ at pH 7.5 in the presence of 10 $\mu$M FSK and various antagonists. cAMP accumulation for each antagonist is normalized to the value at 5% (v/v) $CO_2$ ($n > 4$; *, $p < 0.05$; #, not significant). *D* and *E*, 10 $\mu$M FSK stimulated cAMP accumulation in DT40KO (*D*), and DT40-$IP_3$R1 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-$IP_3$R1 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 $\mu$M 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 $\mu$M 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-$IP_3$R1 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-$IP_3$R1 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-$IP_3$R1 cells as observed for both OK and HEK-PR1 cells.



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-$IP_3$R1 cells. Cytoplasmic [$Ca^{2+}$] was elevated at 10% compared with 5% $CO_2$ in DT40-$IP_3$R1 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-$IP_3$R cells with either 1 mM EGTA, 100 $\mu$M 2-APB, or 500 nM xestospongin C (Fig. 4*F*). Cytoplasmic [$Ca^{2+}$] was elevated at 10% compared with 5% $CO_2$ in DT40-$IP_3$R1 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 $\mu$M) 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 $\mu$M 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 $\mu$M) 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 $\mu$M 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-$IP_3$R1 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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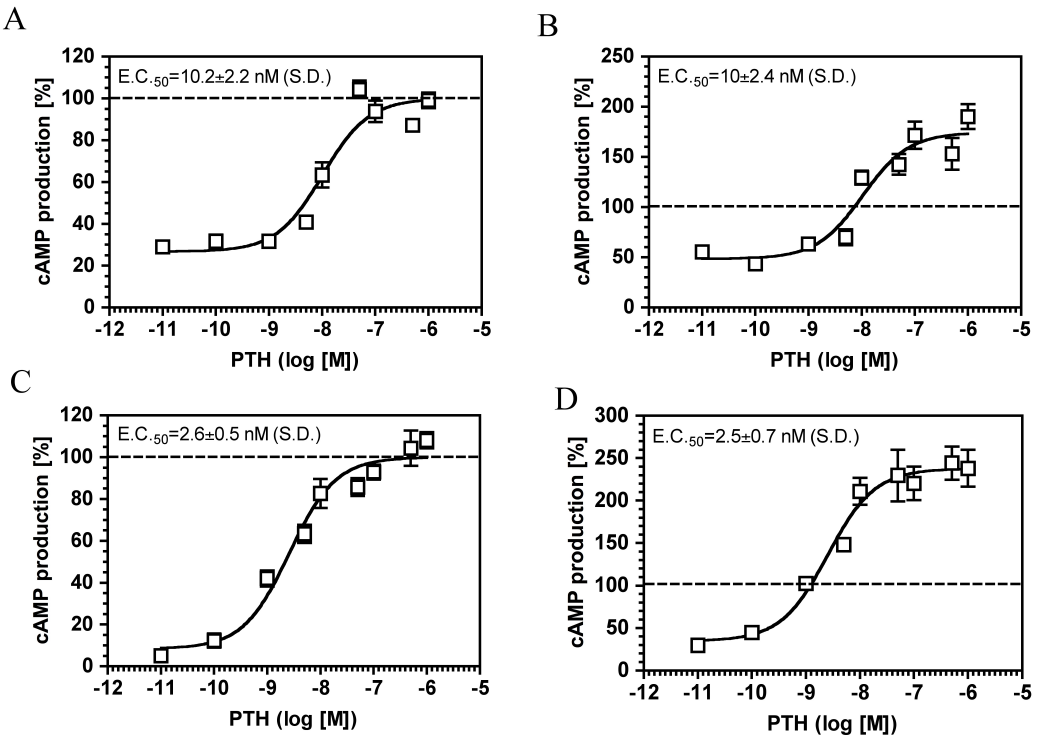
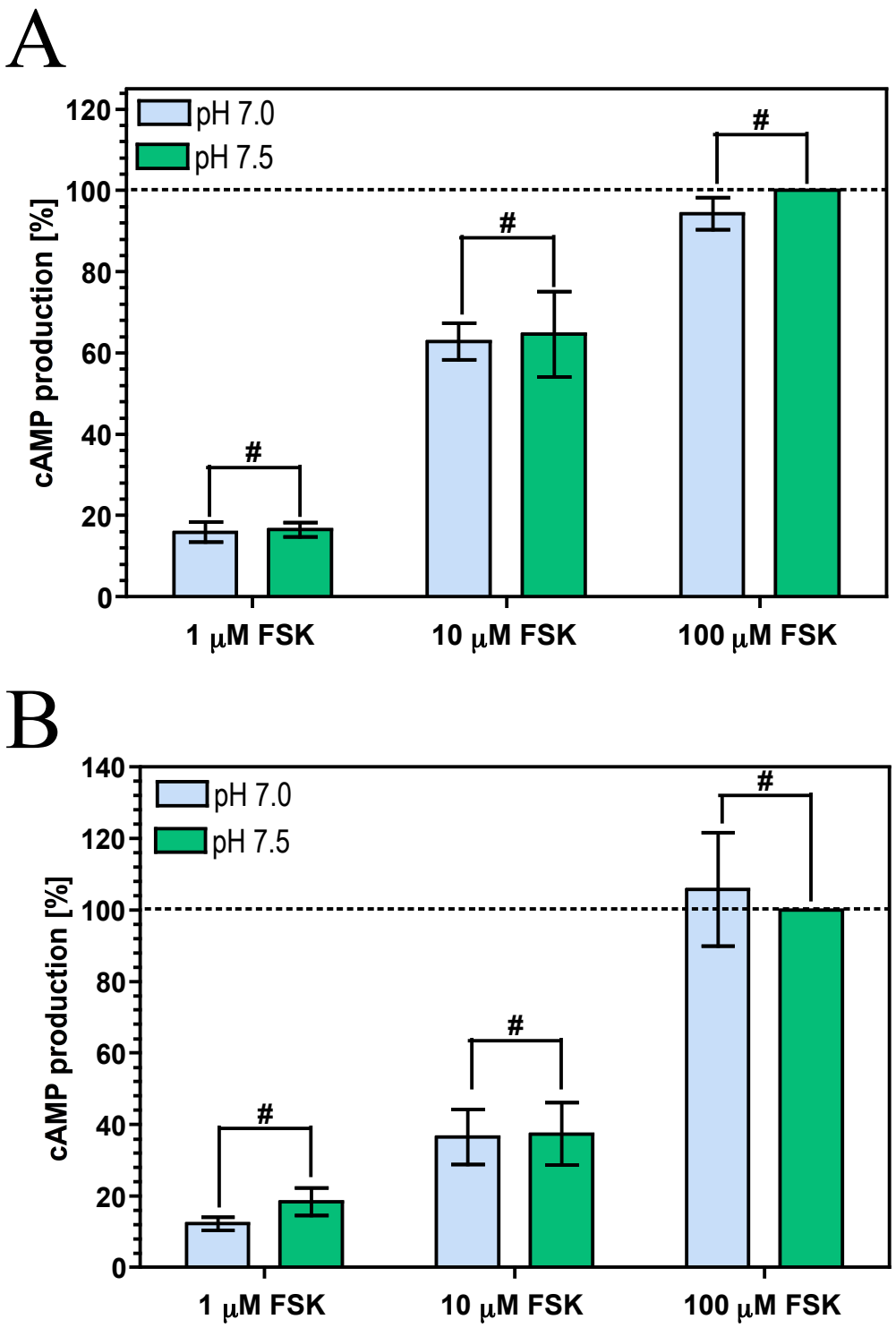
**Supplementary Figure 1.** Extracellular pH does not influence the potency of the PTH receptor-AC signalling complex for PTH. cAMP accumulation in OK (A, B) or HEK-PR1 cells (C, D) at pH 7.5 (A, C) or pH 7.0 (B, D) at varying PTH. cAMP accumulation in each cell line is normalized to the value for the best fit for maximum cAMP accumulation at pH 7.5 ($n > 4$).



**Supplementary Figure 2.** Extracellular pH does not influence the efficacy or potency of the forskolin mediated cAMP response. cAMP accumulation in OK ($n$ = 4, # not significant) (A) or HEK-PR1 cells ($n$ = 3, # not significant) (B) at pH 7.0 or 7.5 at varying forskolin (FSK). cAMP accumulation in each cell line is normalized to the response to 100 μM forskolin at pH 7.5.



**Supplementary Figure 3.** Elevated $CO_2$ reduces cAMP accumulation in UMR-106 cells. cAMP accumulation in UMR-106 cells exposed to 5% (v/v) $CO_2$ or 10% (v/v) $CO_2$ at pH 7.5 (A, C) and pH 7.0 (B, D) in the presence of 5 nM PTH (A, B) or 10 μM FSK (C, D). cAMP accumulation in each graph is normalized to the value in the presence of agonist at 5% (v/v) $CO_2$ ($n > 4$, * $p < 0.05$).