Elevated Carbon Dioxide Blunts Mammalian cAMP Signaling Dependent on Inositol 1,4,5-Triphosphate Receptor-mediated Ca\(^{2+}\) Release\(^{\text{a,b}}\)

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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 acidic 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 carboxic 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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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$_{R1}$ 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 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 was measured using a microspectrofluorometric system (excitation, 490/440 nm; emission, 535 nm). pH 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$^{-1}$ [3H]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 the twocolumn chromatography (38). DT40KO and DT40-IP$_{R1}$ cell cAMP was assayed using the Biotrak cAMP enzyme immunoassay (GE Healthcare) according to the manufacturer’s 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$_2$O 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 twocolumn method (38).

Measurement of NHE3 Activity—NHE3 activity was monitored by measuring pH recovery after a NH$_4$Cl 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$_4$Cl pulses (110 mM NaCl, 25 mM glucose, 20 mM NH$_4$Cl, 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$_4$Cl.

Ca$^{2+}$ Imaging—Cells were loaded with 10 μM of the Ca$^{2+}$-sensitive fluorescent dye Fura 2-AM in serum-free medium 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)
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\(_2\) on 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, Go\(_i\). 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. 1A) (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. 1B). 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 \pm 0.1 (mean \pm S.D.) and at 10% CO\(_2\) was 7.4 \pm 0.1 (mean \pm 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 \mu M forskolin (FSK), indicating that the effect of CO\(_2\) is independent of the mechanism used to stimulate AC (Fig. 1C). 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 acidification caused by CO\(_2\) (\(\Delta\text{pH}_{i} = -0.72 \pm 0.17\); Fig. 1D). We approximated the extent of intracellular acidification with media containing 2 mm propionic acid (\(\Delta\text{pH}_{i} = -0.60 \pm 0.35\); Fig. 1F). 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. 1F). 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. 1G). 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. 1H). We examined whether the effect of CO\(_2\) on AC activ-

- 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 \(p < 0.05\).
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ity was due to protein degradation. The in vitro AC activity of OK cell crude membrane preparations exposed to 5 or 10% CO\textsubscript{2} was similar (the specific activity at 10% CO\textsubscript{2} was 115 ± 8% (S.E., n = 6) that at 5% CO\textsubscript{2}), indicating similar protein levels and consistent with the reversibility of the response to CO\textsubscript{2}.

To determine whether the CO\textsubscript{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 mM PTH was reduced at elevated CO\textsubscript{2} independent of extracellular pH (Fig. 2, A and B, supplemental Fig. S1) and any specific pathway used to stimulate AC (10 \mu M FSK; Fig. 2C and supplemental Fig. S2). 2 mM propionic acid gave a drop in pH (ΔpH = −0.61 ± 0.32; Fig. 2E) greater than that for elevated CO\textsubscript{2} (ΔpH = −0.21 ± 0.14; Fig. 2D) but had no influence on cAMP accumulation (Fig. 2F). Similar to OK cells, propionic acid did not influence the response of cAMP to CO\textsubscript{2} (Fig. 2G). As the experimental process of elevating CO\textsubscript{2} in air causes a small hypoxic effect (from 19.9% to 18.9% O\textsubscript{2} (v/v)) we examined whether a shift from 20% to 18% O\textsubscript{2} at constant CO\textsubscript{2} influenced cAMP levels as an additional control. The mild hypoxia had no influence on cAMP indicating that the effect is mediated through CO\textsubscript{2} (Fig. 2H).

Similar to OK cells, the in vitro AC activity of HEK-PR1 crude membrane preparations exposed to 5 or 10% CO\textsubscript{2} was similar (the specific activity at 10% CO\textsubscript{2} was 99 ± 5% (S.E., n = 5) that at 5% CO\textsubscript{2}) indicating similar protein levels and cells grown at 5% CO\textsubscript{2} and then exposed to 10% CO\textsubscript{2} prior to assay at 5% CO\textsubscript{2} with FSK demonstrated cAMP accumulation indistinguishable from cells maintained and assayed at 5% CO\textsubscript{2} (Fig. 2F). To confirm that the effect of elevated CO\textsubscript{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\textsubscript{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\textsubscript{2}. We used antagonists whose broad target range enabled us to simultaneously inhibit multiple cAMP interacting signaling pathways (Fig. 3A). The effect of CO\textsubscript{2} on cAMP accumulation in HEK-PR1 cells did not require the activity of cAMP phosphodiesterase (1 mm 3-isobutyl-1-methylxanthine), soluble adenyl cyclase (10 \mu M KH7), cAMP-dependent protein kinase (PKA) (10 \mu M H-89), calcium-calmudulin-dependent protein kinase II (100 nm autocamtide II), or protein kinase C (1 \mu M staurosporine/1 mm Gö 6983). The lack of an effect of CO\textsubscript{2} with the AC inhibitor SQ 22,536 (200 \mu M) 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 \mu M acetazolamide) had no effect, indicating no requirement for conversion of CO\textsubscript{2} to HCO\textsubscript{3}− (14). 1 mm extracellular ethylene glycol tetaacetic acid (EGTA) had no effect on the CO\textsubscript{2} response, whereas it was ablated by the acetoxymethyl ester of 1,2-bis(2-amino-phenoxy)ethane-N,N′,N′′,N′′′-tetaacetic acid (BAPTA-AM; 1 mm), indicating a requirement for intracellular but not extracellular Ca\textsuperscript{2+}. The influence of BAPTA-AM on the CO\textsubscript{2} response was confirmed in OK cells, demonstrating a likely common mechanism of action (Fig. 3B). We used further inhibitors to investigate the source of the intracellular Ca\textsuperscript{2+}. The CO\textsubscript{2} effect was insensitive to 100 \mu M nifedipine (L- and T-type voltage-dependent Ca\textsuperscript{2+} channel blocker) in both OK and HEK-PR1 cells and 5 \mu M rotenone (a mitochondrial inhibitor) in HEK-PR1 cells (Fig. 4, A and B). Rotenone ablated the response of cAMP to CO\textsubscript{2} in OK cells; however, we noted sig-
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 CO₂ mediated Ca^{2+} release from a thapsigargin-sensitive store, most likely via the IP₃ receptor and the endoplasmic reticulum. Further evidence for this was obtained in HEK-PR1 cells using the IP₃ R inhibitor 100 μM 2-APB (Fig. 4C). 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 IP₃ receptor in Ca^{2+} release. We examined the effect of elevated CO₂ on the DT40KO cell line (48). DT40KO cells are a chicken B lymphocyte-derived cell line genetically ablated for type 1, 2, and 3 IP₃ receptors and are a null background for IP₃ receptor studies. We examined cAMP accumulation in response to elevated CO₂ in DT40KO cells compared with DT40-IP₃ R1 cells that have had the rat IP₃ type 1 receptor introduced (49). Elevated CO₂ did not blunt cAMP accumulation in DT40KO cells (Fig. 4D) but did in DT40-IP₃ R1 cells (Fig. 4E), proving that the type 1 IP₃ receptor is required for the response to CO₂. Intracellular acidification through 2 mM propionic acid had no influence on cellular cAMP in DT40-IP₃ R1 cells as observed for both OK and HEK-PR1 cells.

Figure 4: Ca²⁺ release via IP₃ R is required for CO₂ to reduce cellular cAMP. A–C, cAMP accumulation in HEK-PR1 (A and C) or OK (B) cells exposed to 5% (v/v) CO₂ or 10% (v/v) CO₂ 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) CO₂ (n > 4; *, p < 0.05; #, not significant). D and E, 10 μM FSK stimulated cAMP accumulation in DT40KO (D), and DT40-IP₃ R1 cells (E) exposed to 5% (v/v) CO₂. 10% (v/v) CO₂, or 5% (v/v) CO₂ with 2 mM propionic acid (PA) at pH 7.5. cAMP accumulation in each graph is normalized to the value at 5% (v/v) CO₂ (n > 3; *, p < 0.05; #, not significant). F, ratio of cytosolic Ca²⁺ at a test condition versus 5% (v/v) CO₂ at pH 7.5 in DT40-IP₃ R1 or DT40-KO cells in the presence of various antagonists (n > 6; *, p < 0.05; #, not significantly <10% (v/v) CO₂ by one-way analysis of variance with post-hoc one-sided Dunnett test).
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As the CO₂/cAMP effect is sensitive to intracellular Ca²⁺ chelation and shows a requirement for the IP₃ receptor, we investigated whether elevated CO₂ altered steady state cytoplasmic [Ca²⁺] in both DT40KO and DT40-IP₃R1 cells. Cytoplasmic [Ca²⁺] was elevated at 10% compared with 5% CO₂ in DT40-IP₃R1 but not DT40KO cells (Fig. 4F). Nonspecific intracellular acidification through propionic acid had no influence on cytoplasmic [Ca²⁺]. Elevated CO₂ therefore mediates Ca²⁺ release from the endoplasmic reticulum via the IP₃ receptor. To provide an independent validation for the role of the IP₃ receptor in the DT40 cell response to CO₂, we treated DT40-IP₃R cells with either 1 mM EGTA, 100 μM 2-APB, or 500 nM xestospongin C (Fig. 4F). Cytoplasmic [Ca²⁺] was elevated at 10% compared with 5% CO₂ in DT40-IP₃R1 cells in the presence of EGTA, consistent with the absence of an effect of EGTA on CO₂ modulation of cAMP. The Ca²⁺ release in response to CO₂ was ablated by the IP₃ receptor antagonists xestospongin C and 2-APB. This result supports the interpretation that these inhibitors are blocking IP₃ receptor signaling in HEK-PR1 and OK cells (Figs. 4C and 5B). Inclusion of the AC inhibitor SQ 22,536 (200 μM) had no influence on CO₂-mediated Ca²⁺ release. These data confirm that an increase in cytosolic Ca²⁺ is a prerequisite for the effect of CO₂ on cAMP (Fig. 4, D and E) and that cAMP lies downstream of the increase in cytosolic Ca²⁺ (Fig. 4F).

We investigated the functional consequences of CO₂-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 CO₂ on cAMP-mediated suppression of NHE3 activity. OK cells exposed to NH₄Cl alkalize due to H⁺ buffering by NH₃, but pH regulatory mechanisms return pH₂O to normal (Fig. 5A). On exchange of NH₄Cl for NaCl, pH₂O drops as the accumulated intracellular NH₄⁺ releases H⁺. The alkalization to restore pH₂O is due to NHE3 (52), and we analyzed this phase of the response.

Comparison of control pH₂O recoveries at 5 and 10% CO₂ demonstrated a cAMP-independent suppression of recovery at elevated CO₂ (Fig. 5, A and B; note the ratio of recovery at 10% compared with 5% CO₂ < 1). Inhibition of NHE3-mediated pH₂O recovery by FSK or PTH was greater in 5% compared with 10% CO₂ consistent with the effect of CO₂ on cAMP levels (note the ratio of recovery at 10% CO₂ compared with 5% CO₂ > 1). The effect of CO₂ on cAMP inhibition of pH₂O recovery was reduced by H-89 and BAPTA-AM, demonstrating a requirement for both PKA and intracellular Ca²⁺. 10 μM dantrolene (a ryanodine receptor antagonist) had no influence on CO₂ suppression of cAMP signaling. The IP₃ receptor antagonists xestospongin C (500 nM) and 2-APB (100 μM) eliminated the effect of CO₂ on cAMP-dependent NHE3 inhibition. CO₂ therefore suppresses the activity of the cAMP signaling pathway through Ca²⁺ release via the IP₃ receptor with functional consequences for cAMP-dependent cellular processes (Fig. 5C).

**DISCUSSION**

In this work, we make two original claims. The first is that molecular CO₂ 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 CO₂, increase. The second is that molecular CO₂ increases steady state cytoplasmic Ca²⁺ concentrations dependent on the IP₃ receptor. The effect of CO₂ on cAMP is a consequence of this altered Ca²⁺. These findings significantly advance our understanding of the effects of CO₂ on the cell.

CO₂ 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 CO₂ blunts cellular cAMP production independent of pH₂O. 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 pH₂O in OK cells (supplemental Fig. S1). As the assay procedure used has minimal effects on pH₂O, this issue with this batch of cells does not affect the main findings. Cell batches used to examine the influence of CO₂ 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 CO₂ are mediated by pH₂O. First, pH₂O is allowed to normalize after CO₂ 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 CO₂ arguing against a localized acid signal communicating with a distinct signaling enzyme. Extracellular pH₂O is also unlikely to be responsible as medium acidification (as might be proposed to occur mid-assay) were buffering insufficient) does not alter the EC₅₀ for PTH and actually gives an increase in cAMP. Medium acidification would therefore cause an under-estimation of the decrease in cAMP. An increase in pCO₂ causes a small decrease in pO₂, but the decrease in pO₂ did not explain the effects of pCO₂ on cAMP. We conclude, therefore, that the effect of hypercapnia is mediated by molecular CO₂ and not pH₂O or any other variable.

The influence of CO₂ 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-
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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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**REFERENCES**

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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).