The Journal of Physiology

http://jp.msubmit.net

JP-RP-2015-271309R2

Title: Hypercapnia Modulates cAMP Signalling and Cystic Fibrosis Transmembrane Conductance Regulator-dependent Anion and Fluid Secretion in Airway Epithelia

Authors: Mark Turner
Vinciane Saint-Criq
Waseema Patel
Salam Ibrahim
Bernard Verdon
Christopher Ward
James Garnett
Robert Tarran
Martin Cann
Michael Gray

Author Conflict: No competing interests declared

Author Contribution: Mark Turner: Collection and assembly of data; Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required)
Vinciane Saint-Criq: Collection and assembly of data; Data analysis and interpretation; Final approval of manuscript (required)
Waseema Patel: Collection and assembly of data; Data analysis and interpretation; Final approval of manuscript (required)
Salam Ibrahim: Collection and assembly of data; Final approval of manuscript (required)
Bernard Verdon: Collection and assembly of data; Final approval of manuscript (required)
Christopher Ward: Provision of study materials or patients; Final approval of manuscript (required)
James Garnett: Provision of study materials or patients
Robert Tarran: Final approval of manuscript (required)
Martin Cann: Conception and design; Provision of study materials or patients; Manuscript Writing; Final approval of manuscript (required)
Michael Gray: Conception and

Disclaimer: This is a confidential document.
Running Title: Hypercapnia modulates cAMP signalling in human airway epithelia

Dual Publication: No

Funding: Medical Research Council (MRC): Mark John Turner; Biotechnology and Biological Sciences Research Council (BBSRC): Waseema Patel; Higher Committee for Education Development (HCED), Iraq: Salam Haji Ibrahim; Cystic Fibrosis Trust: Vinciane Saint-Criq, SRC003

Disclaimer: This is a confidential document.
Hypercapnia Modulates cAMP Signalling and Cystic Fibrosis Transmembrane Conductance Regulator-dependent Anion and Fluid Secretion in Airway Epithelia

Mark J. Turner¹,², Vinciane Saint-Criq¹, Waseema Patel¹, Salam H. Ibrahim¹, Bernard Verdon¹, Christopher Ward², James P. Garnett¹, Robert Tarran³, Martin J. Cann⁴ and Michael A. Gray¹

¹ Institute for Cell & Molecular Biosciences, The Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne, U.K., NE2 4HH.
² Institute for Cellular Medicine, The Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne, U.K., NE2 4HH.
³ Marsico Lung Institute, University of North Carolina, Chapel Hill, North Carolina, USA, 27599.
⁴ School of Biological and Biomedical Sciences, Durham University, South Road, Durham, U.K., DH1 3LE.
⁵ Department of Physiology, McIntyre Medical Sciences Building, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec, Canada, H3G 1Y6.

To whom correspondence should be addressed: Dr. Michael Gray, Institute for Cell & Molecular Biosciences, The Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, U.K. Tel: 0191 208 7592. Fax: 0191 208 7424. E-mail: m.a.gray@ncl.ac.uk.

Running head: Hypercapnia modulates cAMP signalling in human airway epithelia

Key words: carbon dioxide, cAMP, CFTR
Key Points

- Raised arterial blood CO\(_2\) (hypercapnia) is a feature of many lung diseases.
- CO\(_2\) has been shown to act as a cell signalling molecule in human cells, notably by influencing the levels of cell signalling second messengers: cAMP and Ca\(^{2+}\).
- Hypercapnia reduced cAMP-stimulated CFTR-dependent anion and fluid transport in Calu-3 cells and primary human airway epithelia but did not affect cAMP-regulated HCO\(_3^-\) transport via pendrin or Na\(^+/\)HCO\(_3^-\) cotransporters.
- These results further support the role of CO\(_2\) as a cell signalling molecule and suggests CO\(_2\)-induced reductions in airway anion and fluid transport may impair innate defence mechanisms of the lungs.

Abstract

Hypercapnia is clinically defined as an arterial blood partial pressure of CO\(_2\) of above 40\(\text{mmHg}\) and is a feature of chronic lung disease. In previous studies we have demonstrated that hypercapnia modulates agonist-stimulated cAMP levels through effects on transmembrane adenylyl cyclase activity. In the airways, cAMP is known to regulate cystic fibrosis transmembrane conductance regulator (CFTR)-mediated anion and fluid secretion, which contributes to airway surface liquid homeostasis. The aim of the current work was to investigate if hypercapnia could modulate cAMP-regulated ion and fluid transport in human airway epithelial cells. We found that acute exposure to hypercapnia significantly reduced forskolin-stimulated elevations in intracellular cAMP as well as both adenosine and forskolin-stimulated increases in CFTR-dependent transepithelial short-circuit current, in polarised cultures of Calu-3 human airway cells. This CO\(_2\)-induced reduction in anion secretion was not due to a decrease in HCO\(_3^-\) transport given that neither a change in CFTR-dependent HCO\(_3^-\) efflux, nor Na\(^+/\)HCO\(_3^-\) cotransporter-dependent HCO\(_3^-\) influx were CO\(_2\)-sensitive. Hypercapnia also reduced the volume of forskolin-stimulated fluid secretion over 24 hours, yet had no effect on the HCO\(_3^-\) content of the secreted fluid. Our data reveal that hypercapnia reduces CFTR-dependent, electrogenic Cl\(^-\) and fluid secretion, but not CFTR-dependent HCO\(_3^-\) secretion, which highlights a differential sensitivity of Cl\(^-\) and HCO\(_3^-\) transporters to raised CO\(_2\) in Calu-3 cells. Hypercapnia also reduced forskolin-stimulated CFTR-dependent anion secretion in primary human airway epithelia. Based on current models of airways biology, a reduction in fluid secretion, associated with hypercapnia, would be predicted to have important consequences for airways hydration and the innate defence mechanisms of the lungs.

Abbreviations List: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; I\(_{sc}\), short circuit current; NBC, Na\(^+/\)HCO\(_3^-\) cotransporter; NHE, Na\(^+/\)H\(^+\) exchanger; pH\(_i\), intracellular pH; pH\(_e\), extracellular pH; PKA, protein kinase A; sAC, soluble adenylyl cyclase; tmAC, transmembrane adenylyl cyclase; V\(_{te}\), transepithelial voltage.
Introduction
Carbon dioxide constitutes 0.04% by volume of the Earth’s atmosphere (van der Laan-Luijkx et al., 2013) and has major roles in plant, prokaryote and animal biology (Cummins et al., 2014). In plants, CO₂ is used to synthesize sugars during photosynthesis whilst in animals, although CO₂ is a waste product of cellular respiration, it also has an important role in maintaining plasma pH via its buffering effect on HCO₃⁻ (Marques et al., 2003) as well as stimulation of peripheral and central chemoreceptors to regulate ventilation (Somers et al., 1989; Guyenet et al., 2010). Elevated CO₂ in arterial blood (hypercapnia) is associated with lung disease in humans (Lourenço & Miranda, 1968; Prin et al., 2002), yet the effects of hypercapnia in human physiology are not fully understood. In mammals, recent studies have provided strong evidence that CO₂ can act as a bona fide cell signalling molecule, and that changes in CO₂ alter the activity of a variety of membrane transporters, including connexin 26 (Huckstepp et al., 2010a; Huckstepp et al., 2010b; Meigh et al., 2013), the epithelial Na⁺/HCO₃⁻ cotransporter (NBC) (Adjiano et al., 2009), inwardly rectifying K⁺ channels (Huckstepp & Dale, 2011) and the Na⁺/K⁺-ATPase (Briva et al., 2007; Vadasz et al., 2008). The action of CO₂ on membrane transporters has been shown to involve different mechanisms. For instance, CO₂-dependent downregulation of Na⁺/K⁺-ATPase activity specifically involves the endocytosis of the α subunit of the Na⁺/K⁺-ATPase, demonstrating that CO₂ can alter surface expression of ion transporters (Briva et al., 2007). Alternatively, CO₂ directly modulates connexin 26 via carbamylation, a post-translational modification whereby a covalent bond forms between the carbon in CO₂ and a primary amine group of the target protein (Meigh et al., 2013). In addition, CO₂ also has reported effects on key cell second messengers involved in membrane transporter regulation; specifically cyclic AMP and Ca²⁺ (Cann et al., 2003; Cann, 2004). cAMP is synthesized from ATP, a reaction catalysed by adenylyl cyclase (tmAC) and the soluble adenylyl cyclase (sAC) in mammals (Buck et al., 1999). Our laboratory have previously shown that the activity of a recombinant, catalytically active mammalian tmAC, expressed in HEK 293T cells, was significantly higher in cells exposed to 5% CO₂ compared to those exposed to 0.03% CO₂, demonstrating that tmAC is sensitive to changes in CO₂ (Townsend et al., 2009). This study also showed that tmAC was sensitive to CO₂ but not HCO₃⁻ in vivo and in vitro, supporting previous findings that first proposed tmAC activity was only sensitive to CO₂ and not inorganic carbon per se (Hammer et al., 2006). More recently, we have shown that incubating OK cells (a model of human proximal tubule cells) in 10% CO₂ caused a significant reduction in forskolin and parathyroid hormone-stimulated increases in intracellular cAMP ([cAMP]ₖ) compared to levels measured under normocapnic conditions of 5% CO₂ (Cook et al., 2012). The decrease in cAMP correlated with an enhanced activity of the Na⁺/H⁺ exchanger (NHE) 3, a transporter known to be negatively regulated by cAMP/PKA, thus providing evidence that hypercapnia was able to modulate cAMP-regulated transporters in human epithelial cells. This work further showed that the effect of raised CO₂ on cAMP was dependent on an IP₃-dependent release of Ca²⁺ which, in turn, led to an inhibition in tmAC activity, thereby demonstrating that CO₂ affected Ca²⁺ as well as cAMP signalling. These data supported earlier studies that demonstrated CO₂ modulated Ca²⁺ signalling in other mammalian and human cells (Nishio et al., 2001; Bouyer et al., 2003; Briva et al., 2011).

In the airways, cAMP plays a major role in regulating the volume and composition of the airway surface liquid (ASL). In the upper airways, ASL secretion occurs predominantly from serous cells of the submucosal glands (SMGs). Studies on intact SMG secrections as well as SMG-derived secretory cell lines, such as Calu-3, have found that elevations in intracellular cAMP stimulate CFTR-dependent Cl⁻, HCO₃⁻ and fluid transport (Lee et al., 1998; Devor et al., 1999; Joo et al., 2002; Krouse et al., 2004; Ballard et al., 2006; Ianowski et al., 2007; Lee & Foskett, 2010; Garnett et al., 2011; Huang et al., 2012; Shan et al., 2012). Efficient anion secretion in the airways is paramount in order to maintain ASL hydration and pH, as well as efficient mucus secretion and expansion (Garcia et al., 2009; Chen et al., 2010; Gustafsson et al., 2012; Ridley et al., 2014). Loss of functional expression of CFTR at the apical membrane of HCO₃⁻ secreting epithelia underlies the hereditary disease Cystic Fibrosis (CF) and airways dehydration and impaired ASL alkalination have been reported in CF airways (Coakley et al., 2003; Song et al., 2006; Boucher, 2007) consistent with a key role for CFTR in mediating airway HCO₃⁻ secretion. Furthermore, it has been shown that the acidic ASL found in CF pigs, compromises the ability to kill airway pathogens (Pezzulo et al., 2012) and provides a plausible explanation as to why CF patients are susceptible to airway bacterial colonization.
Given the previously reported findings from our laboratory that hypercapnia modulated cAMP signalling in renal epithelial cells (Cook et al., 2012), we hypothesised that hypercapnia would also affect airway epithelial cell function. Our results show that hypercapnia reduced cAMP levels in Calu-3 cells and this correlated with a drop in cAMP-dependent anion secretion. The reduction in anion secretion appeared primarily due to a reduction in Cl⁻ transport, given that both CFTR-dependent HCO₃⁻ efflux via pendrin, and NBC-dependent HCO₃⁻ import were unaffected by hypercapnia. Furthermore, hypercapnia also reduced the volume of cAMP-stimulated fluid secretion without affecting the HCO₃⁻ content of the fluid, implying Cl⁻ secretion and HCO₃⁻ secretion have differential sensitivities to hypercapnia. Hypercapnia also reduced cAMP-stimulated anion secretion in primary human bronchial epithelial layers, indicating this effect of CO₂ would be predicted to occur in vivo. Our results therefore demonstrate that CO₂ acts as a signalling molecule in human airway epithelia to downregulate anion and fluid secretion.

Materials and Methods

**Calu-3 cell culture:** The human serous cell line, Calu-3 (Shen et al., 1994), were grown in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% (v/v) FCS, 1% (v/v) non-essential amino acids, 2mM L-Glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cells were incubated at 37°C in humidified air containing 5% (v/v) CO₂ and were used between passage 20-50. Unless otherwise stated, 250,000 cells were seeded onto either 12mm Costar Transwells or 12mm Snapwells, 0.4µm pore, polyester membrane inserts, and grown under submerged conditions with 500µl growth media applied to the apical compartment of membrane inserts. The transepithelial electrical resistance (TEER) was routinely measured using an epithelial voltohmmeter (WPI, UK) and cells generally reached a confluent monolayer, with a TEER of above 600Ω cm² after 6 days growth on Transwell inserts. Experiments were performed 9-13 days post seeding.

**Primary human bronchial epithelial cell culture:** Ethical approval was granted for this work from Newcastle and North Tyneside 2 [Min Ref: 2001/179]. Differentiated primary bronchial epithelial cells were derived from bronchial brushings taken from lung transplant recipients during surveillance bronchoscopy as previously described (Forrest et al., 2005). These were grown in a CO₂ incubator (37°C; 5% CO₂) to 90% confluence using Bronchial Epithelial Growth Medium with supplements (BEGM, Lonza) in T25 flasks pre-coated with 32µg/mL collagen. Cells were passaged using standard trypsin/EDTA technique and cryopreserved for future use. After reconstitution, cells were once again expanded to near confluence in T25 flasks, before being seeded onto collagen-coated 12 mm Costar Snapwells at a density of 100,000 cells per membrane in 0.5 mL BEGM, with 2 mL of this medium applied to the basal chamber. Confluence was reached after 72 hr, at which point the cell culture was taken to air-liquid interface (ALI). Here, the medium above the cells was removed completely, and the cells were subsequently fed only from the basal chamber with an ALI medium as described by Fulcher et al. (Fulcher et al., 2005). Ciliogenesis was first observed at 14 days after ALI, and short-circuit current measurements were performed 30–35 days post growth at ALI.

**Short-circuit current measurements:** Cells were grown on Snapwell inserts and mounted into an Ussing chamber in which each chamber was connected to a calomel voltage sensing electrode and an AgCl₂ current sensing electrode by 3M KCl salt bridges containing 3% (w/v) agar. Cells were bathed in 7.5mls of Krebs solution and continually gassed with either 5% (v/v) CO₂/95% (v/v) O₂ for control conditions or 10% (v/v) CO₂/90% (v/v) O₂ to induce hypercapnia. To measure the short circuit current (Iₑ), cells were clamped at 0mV using a DVC-1000 Voltage/Current Clamp (WPI, Hitchen, UK) and a Powerlab 1200 feedback amplifier (AD Instruments, Oxford, UK) injected the appropriate current to clamp transepithelial voltage (Vₑ) to 0mV which was recorded as the Iₑ using Scope 3 software (AD Instruments). To monitor transepithelial resistance (Rₑ), a 2 s 10mV pulse was applied every 30 s.

**Intracellular pH measurements:** Calu-3 cells were grown on Transwell inserts and loaded with the pH-sensitive, fluorescent dye BCECF-AM (10µM) for one hour in a NaHEPES buffered solution at 37°C. Cells were mounted on to the stage of a Nikon fluo inverted microscope and perfused with a modified Krebs solution gassed with either 5% (v/v) CO₂/95% (v/v) or O₂ 10% (v/v) CO₂/90% (v/v) O₂. Solutions were perfused across the apical and basolateral membranes at 37°C at a speed of 3ml min⁻¹ (apical) and 6ml min⁻¹ (basolateral). Intracellular pH (pHᵢ) was measured using a Life Sciences Microfluorimeter System in which cells were alternatively excited at 490nm and 440nm.

4
wavelengths every 1.024 s with emitted light collected at 510 nm. The ratio of 490 nm emission to 440 nm emission was recorded using PhoCal 1.6b software and calibrated to pH$_i$ using the high K’/nigericin technique (Hegyi et al., 2003) in which cells were exposed to high K’ solutions containing 10 µM nigericin, set to a desired pH, ranging from 6.6 to 8.4. Total buffering capacity ($\beta_{tot}$) was calculated by addition of the intrinsic buffering capacity ($\beta_i$) to the buffering capacity of the CO$_2$-HCO$_3$ buffer system ([$\beta$HCO$_3$]) in which $\beta_i$ was calculated using the NH$_4^+$ technique as described by Roos and Boron (1981). For analysis of pH$_i$ measurements, delta pH$_i$ ($\Delta$PHi/\Delta t$) was determined by calculating the mean pH$_i$ over 60 s resulting from treatment. Rate of pH$_i$ change ($\Delta$PHi/\Delta t$) was determined by performing a linear regression over a period of at least 30 s which was converted to a transmembrane HCO$_3$ flux (-J(B)) by multiplying $\Delta$PHi/\Delta t by $\beta_{tot}$.

**Radiolabelled cAMP assay:** Calu-3 cells were cultured in Corning 12 well plates at an initial seeding density of 3 x 10$^5$ cells/well and used at approximately 80% confluency. Cells were loaded with 2µCi ml$^{-1}$ [3H]-adenine and incubated for 2 hours at 37°C in humidified air containing 5% (v/v) CO$_2$. Cells were then washed twice with PBS and incubated for a further 30 minutes at 37°C in humified air containing 5% (v/v) CO$_2$/95% (v/v) O$_2$ (normocapnic controls) or 10% (v/v) CO$_2$/90% (v/v) O$_2$ (hypercapnia). Incubation was performed in growth medium containing 1mM IBMX that had been pregassed with the appropriate CO$_2$ concentration and titrated to pH 7.4 using 1M NaOH. Forskolin (5µM) was then added to the cells for 10 minutes before the assay was ended by removal of media and lysis of cells by adding 5% (w/v) trichloroacetic acid containing 1mM ATP and 1mM cAMP for one hour at 4°C. cAMP levels in lysates were measured by the twin column chromatography procedure described by Johnson et al. (1994).

**Cell surface biotinylation:** Calu-3 cells were grown on Transwell inserts and washed three times with PBS. Cells were then incubated at 37°C in humidified air containing 5% (v/v) CO$_2$ (control) or 10% (v/v) CO$_2$ (hypercapnia) in pregassed high Cl Krebs solution for 20 mins. The solution was removed and cells were incubated for 30 minutes at 4°C in PBS++ (PBS containing 0.1mM Ca$^{2+}$ and 1mM Mg$^{2+}$; pH 8.0) with 0.5mg/ml EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) added to the apical membrane. Biotinylation was stopped by removal of the apical solution and addition of ice cold PBS++. Cells were then lysed using RIPA buffer containing 150mM NaCl, 20mM Tris, 1% Triton-X-100, 0.1% SDS and 0.08% sodium deoxycholate (pH8.0) with 1 protease inhibitor cocktail tablet (Roche Applied Sciences) added to 50ml of RIPA buffer. The lysate was collected and centrifuged for 15 mins at 13,000 RPM at 4 degrees and the protein concentration of the supernatant was assessed using the BCA protein assay kit (Pierce Biotechnology Inc.). 100µg of protein was taken to be used for analysis of whole cell protein expression. Streptavidin agarose beads (Novagen) that had been equilibrated with PBS++ and RIPA buffer were added to the remaining protein at 1µl beads/20µg protein and incubated overnight at 4 degrees with continuous inversion of samples to ensure thorough mixing. These samples were then centrifuged and washed 5 times with RIPA buffer and heated to 65°C for 5 minutes. Protein expression was then detected by Western blot.

**Western blot:** SDS-PAGE using 7% gels was performed on all samples at 120V for 2 hours. Samples were then transferred to a nitrocellulose membrane at 400mA for 1 hour 30 minutes at 4°C. The membrane was blocked for one hour in blocking buffer consisting of TBS (Tris Buffered Saline) + 0.1% Tween 20 (TTBS) containing 5% dried skimmed milk powder (Compliments) before primary mouse anti-CFTR monoclonal antibody 23C5 (1:200 dilution in TBS) and mouse anti-$\alpha$ tubulin antibody (1:1000 dilution in TBS) were added overnight at 4°C. The membrane was then washed using TTBS before a goat anti-mouse antibody labelled with horse radish peroxidase (HRP) was added at 1:5000 dilution in TBS for one hour. Any unbound secondary antibody was then washed off with TTBS. To detect any HRP activity, equal volumes of the enhanced chemiluminescent substrates Enhanced Luminol Reagent and the Oxidizing Reagent (Thermo Scientific) were added to the blot for 10 minutes before the blot was exposed to Kodak Scientific Imaging film for 30 seconds. The film was developed and the band intensity was analysed using ImageJ software.

**Fluid secretion assays:** Calu-3 cells were grown on Transwell inserts and washed three times with PBS in order to remove any mucus that may have accumulated over time. Extra care was taken when removing the PBS to ensure no residual fluid remained in the transwell at the end of the washes. Solutions were then added to the cells (1ml basolaterally, 200µl apically) and cells were incubated at 37°C in humidified air containing 5% (v/v) CO$_2$ (control) or 10% (v/v) CO$_2$ (hypercapnia) for 24 hours (Garnett et al., 2011). The apical fluid was then removed and its volume measured. 180µl was
removed first and then the rest of the fluid was removed 1µl at a time to ensure high accuracy. Samples were collected in an Eppendorf tube and after a full equilibration in either 5 or 10 % CO₂, had the pH assessed using a MiniFrod electrode (Hamilton, Reno, USA). This enabled the HCO₃⁻ concentration of the secreted fluid to be calculated using the Henderson-Hasselbalch equation, where: pH = pKₐ + log₁₀ ([HCO₃⁻]/[0.03 x pCO₂]) where pKₐ = 6.1 (the negative log of the carbonic acid dissociation constant).

Periodic acid-Schiffs (PAS) Assay: Given it has been reported that Calu-3 cells secrete mucins, notably MUC5AC (Kreda et al., 2007; Kreda et al., 2010), the PAS assay was used to detect the glycoprotein content of the secreted fluid as an indicator of secreted mucin. To generate a standard curve, pig mucin (a gift from Prof. Jeff Pearson, Newcastle University) was diluted to (in µg/ml) 100, 50, 20, 10, 5, 2 and 1 and 100µl of standards were added to a 96 well plate in duplicate. 100µl of sample was made to 1ml by addition of deionised water and 100µl was added to wells in duplicate. 100µl of a periodic acid/acetic acid mix (made from 10µl periodic acid and 990µl of 50% acetic acid) was added to all standards and samples and the plate incubated for 60 mins at 37°C. 100µl of 1.6% sodium metabisulphate solution in Schiff’s reagent was added to all standards and samples. The plate was then incubated at room temperature for 30 minutes before absorbance was read at 550nm using a BioTek ELx808 Absorbance Microplate Reader. Absorbance was then converted to mucin concentration using the standard curve.

Solutions and reagents: All reagents were purchased from Sigma Aldrich (Poole, UK) apart from forskolin and ouabain (R & D Systems, Abingdon, UK), BCECF-AM (Invitrogen, Paisley, UK) and GlyH-101 and CFTRinh172 (Calbiochem, Watford, UK). All gas cylinders were purchased from BOC and consisted of the following mixtures: 5% CO₂/95% O₂ and 10% CO₂/90% O₂. NaHEPES solution consisted of (in mM) 130 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 NaHEPES and 10 D-Glucose, pH 7.4 at 37°C. High Ca⁺ Krebs solution consisted of (in mM) 25 NaHCO₃, 115 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂ and 10 D-Glucose (pH 7.4). For high Cl⁻, Na⁺ free solutions, NaHCO₃ was replaced with choline bicarbonate and NaCl was replaced with NMDG-Cl. Zero Cl⁻ Krebs solution consisted of (in mM) 25 NaHCO₃, 115 NaGlucanate, 2.5 K₂SO₄, 1 CaGlucanate, 1 MgGlucanate and 10 D-Glucose. Intracellular pH calibration solutions consisted of (in mM) 5 NaCl, 130 KCl, 1 CaCl₂, 1 MgCl₂, 10 D-Glucose, 10 HEPES (for solutions set at pH 7.6 or below) or 10 TRIS (for solutions set at pH 7.8 or above) as well as 10µM nigericin. Solutions used to determine intracellular buffering capacity consisted of (in mM) 4.5 KCl, 1 MgCl₂, 2 CaCl₂, 5 BaCl₂, 10 HEPES, 10 D-Glucose as well as varying concentrations of NH₄Cl/NMDG-Cl, ranging from 0 NH₄Cl/145 NMDG-Cl to 30 NH₄Cl/115 NMDG-Cl. All solutions were titrated to pH 7.4 at 37°C using 1M CsOH.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 4 software. Results are expressed as mean ± S.E.M., of n observations. Student’s t-test, one way ANOVA (with Tukey’s multiple comparison post-test) or two way ANOVA (with Bonferroni post-test) were carried out where applicable to determine statistical significance between measurements. A p value of <0.05 was considered as statistically significant.

Results

Acute hypercapnia attenuates forskolin-stimulated cAMP levels in Calu-3 cells independent of changes in intracellular pH. We first assessed the effect of hypercapnia on the pHᵢ of Calu-3 cells since it is well known that raising CO₂ generally induces cytosolic acidification. Cells were first perfused with Krebs solution gassed with 5% (v/v) CO₂ to maintain cells in a normocapnic environment. Perfusing cells with 10% (v/v) CO₂, caused pHᵢ to decrease by 0.18 ± 0.01 pH units (n=60). This intracellular acidosis recovered after ~20 mins even upon continuous exposure of cells to 10% (v/v) CO₂ (Fig. 1A). We therefore chose 20 mins as an appropriate time to study the effects of acute hypercapnia as cells would have recovered their pHᵢ. Exposure of cells to 10% (v/v) CO₂ for 20 mins did not alter the integrity of the epithelial monolayer as assessed by recording TEER. In normocapnia, TEER was 671 ± 42Ω cm² (n=3) and 600 ± 42Ω cm² in monolayers of Calu-3 cells exposed to acute hypercapnia (p>0.05 vs. normocapnia; n=3). For all experiments, [HCO₃⁻] in the Krebs solution was maintained at 25mM in both normocapnia and hypercapnia. This was necessary to ensure that any effects of hypercapnia on cAMP signalling were due to CO₂-dependent effects on
Forskolin-stimulated transepithelial anion secretion is reduced in conditions of acute hypercapnia in Calu-3 cells. To assess whether the CO₂-induced reductions in forskolin-stimulated [cAMP], modulated cAMP-regulated transepithelial ion transport, ISc measurements were made in monolayers of Calu-3 cells. The ISc is the current required to clamp the transepithelial voltage difference (Vsc) to 0mV. In Calu-3 monolayers, the magnitude of the Vsc is mainly accounted for by transepithelial anion secretion (Lee et al., 1998; Devor et al., 1999; Cobb et al., 2003; Cuthbert et al., 2003; Shan et al., 2012) and therefore changes in ISc reflect changes in anion secretion. Figure 2A shows a representative recording of ISc in normocapnic conditions. To maximize electrogenic Cl⁻ secretion, a basolateral to apical Cl⁻ gradient was applied across the monolayer by reducing apical [Cl⁻] to 40mM by substitution of 84mM NaCl with equimolar NaGluconate. In normocapnia, prior to reducing the apical Cl⁻ concentration, Calu-3 cells displayed a basal ISc of 5.2 ± 0.4µA and further investigations showed that this basal ISc was insensitive to both the basolateral Na⁺/K⁺/2Cl⁻ (NKCC1) inhibitor bumetanide (25µM) and the Na⁺/H⁺ exchanger (NHE) inhibitor EIPA (3µM) (Masereel et al., 2003), whereas application of the CFTR blocker CFTRinh-172 (20µM) reduced basal ISc by 48.5 ± 4.2% (p<0.01; n=3), indicating that the majority of basal ISc was mediated by CFTR. Interestingly, in cells exposed to 20 mins hypercapnia (Fig. 2B), the basal ISc was reduced to 1.3 ± 1.3µA (p<0.01 vs. normocapnia; n=8; Fig. 2C) implying that acute hypercapnia inhibited CFTR-dependent anion secretion under resting conditions. After establishing a basolateral to apical Cl⁻ gradient, addition of forskolin stimulated an increase in ISc which peaked after approximately 90 s to a maximal level and then decreased slightly until a new steady state was reached. The forskolin-stimulated increase in ISc was blocked by a combination of apical CFTRinh-172 (20µM) and basolateral bumetanide (25µM), and both the magnitude and rate of ISc increase were significantly reduced by 61.8 ± 16.0% and 6.8% respectively by the protein kinase A inhibitor H-89 (p<0.05 vs. control; n=3). These results demonstrated that CFTR-dependent anion secretion mediated the forskolin-stimulated increase in ISc, consistent with previous studies (Welsh & Smith, 2001; Kreda et al., 2007; Shan et al., 2012). The maximal forskolin-stimulated increase in ISc (ΔISC) was 19.3 ± 2.0µA cm⁻² (n=10) in normocapnia, compared to 14.1 ± 1.1µA cm⁻² in acute hypercapnia (p=0.053 vs. normocapnia; n=8; Fig. 2D). The rate of forskolin-stimulated increase in ISc in normocapnia was 10.4 ± 1.3µA cm⁻² min⁻¹ (n=10) which was reduced to 5.7 ± 0.6µA cm⁻² min⁻¹ (p<0.01 vs. normocapnia; n=8; Fig. 2E) in cells exposed to acute hypercapnia. These results, combined with those in Fig. 1, imply that attenuation of forskolin-stimulated cAMP levels by acute hypercapnia was sufficient to significantly reduce the rate of cAMP-regulated anion secretion in Calu-3 cells. In addition, the forskolin-stimulated ISc that was sensitive to CFTRinh-172 was also measured. In normocapnia, this was 3.3 ± 0.7µA cm⁻² (n=10) and although it was lower in hypercapnia (1.6 ± 0.2µA cm⁻²; n=8), this was not statistically significant, although a clear trend existed (p = 0.058 vs. normocapnia; Fig. 2F). Taken together with data displayed in Figs. 2C and 2E, these findings suggest CFTR activity is lower in hypercapnia in both basal and forskolin-stimulated conditions.
Acute hypercapnia reduces adenosine but not IBMX-stimulated transepithelial anion secretion in Calu-3 cells. Having shown that hypercapnia reduced forskolin-stimulated I_{sc} in Calu-3 cells, it was important to investigate whether hypercapnia also elicited similar effects when a more physiological agonist was used to increase [cAMP] in Calu-3 cells. For this reason, cells were stimulated with adenosine (Cobb et al., 2003) and the resulting I_{sc} was measured. In normocapnia, adenosine stimulated a maximal I_{sc} increase of 23.9 ± 3.5μA cm^{-2} (n=5) which was significantly reduced to 6.4 ± 1.4μA cm^{-2} in cells exposed to acute hypercapnia (p<0.05 vs. normocapnia; n=3; Fig. 3A). The rate of the adenosine-stimulated increase in I_{sc} was 13.4 ± 8.4μA cm^{-2} min^{-1} (n=5) in normocapnia which was reduced to 2.3 ± 0.8μA cm^{-2} min^{-1} in acute hypercapnia (p = 0.06 vs. normocapnia; n=3; Fig 3B). Therefore, these data demonstrated that hypercapnia reduced adenosine-stimulated, CFTR-dependent anion secretion in Calu-3 cells which mimicked what was observed with forskolin. Interestingly, when [cAMP] levels were increased by stimulation of cells with IBMX, there was no effect of acute hypercapnia on either the IBMX-stimulated ΔI_{sc} (normocapnia = 3.1 ± 0.9μA cm^{-2}; hypercapnia = 3.1 ± 1.3μA cm^{-2}; p>0.05 vs. normocapnia; n=3-4; Fig. 3C) or the rate of IBMX-stimulated increase in I_{sc} (normocapnia = 1.0 ± 0.31μA cm^{-2} min^{-1}; hypercapnia = 1.2 ± 0.8μA cm^{-2} min^{-1} p>0.05 vs. normocapnia; n=3-4; Fig. 3D). Therefore, these data support those observed in Fig. 1B, which demonstrated IBMX-stimulated increases in [cAMP] was insensitive to CO_{2}, and suggest hypercapnia-induced changes in [cAMP] was not due to modulation of IBMX-sensitive PDE activity.

The effect of hypercapnia on cAMP-dependent transepithelial anion secretion is independent of CO_{2}-induced intracellular acidosis: Although I_{sc} measurements performed in hypercapnia were made after 20 mins exposure to 10% CO_{2}, during which time pH_{i} had recovered from intracellular acidosis (as Fig. 1A), it was possible the intracellular acidosis may have induced long term modifications to transporters involved in cAMP-regulated anion secretion. Therefore, cells were acid loaded using 40mM sodium acetate which caused an intracellular acidification of 0.17 ± 0.02 (n=6) that recovered within a 20 min period (Figs. 4A and 4B) and was thus highly similar to the effect of 10% CO_{2}. Thus the effect of forskolin on I_{sc} was measured in cells exposed to 40mM sodium acetate or 80mM mannitol (to compensate for the increased osmolarity of solutions). Representative experiments are shown in figures 4C and 4D. There was no effect of 40mM sodium acetate on either the magnitude or the rate of forskolin-stimulated increases in I_{sc} (Figs. 4E and 4F) and therefore demonstrates that the CO_{2}-induced intracellular acidosis does not contribute to the effects of hypercapnia on cAMP-stimulated anion transport in Calu-3 cells.

Surface expression of CFTR is unaffected by hypercapnia. Our results from the I_{sc} measurements indicated that CO_{2}-induced reductions in [cAMP], were sufficient to reduce cAMP-stimulated, CFTR-dependent anion secretion in Calu-3 cells. To investigate if this observation was due to the effect of CO_{2} on cAMP and not on cell surface levels of CFTR, the amount of CFTR present at the apical membrane was assessed by cell surface biotinylation. Figure 5 shows that after normalizing CFTR levels to α-tubulin, there was no significant effect of CO_{2} on both total cell CFTR expression (p>0.05; n=5 Fig. 5A) or cell surface CFTR expression (p>0.05; n=4 Fig. 5B) which therefore suggest that mechanisms which control CFTR expression at the plasma membrane are insensitive to hypercapnia.

CFTR-regulated, pendrin-dependent apical HCO_{3}^{-} secretion is unaffected by hypercapnia. Having identified that hypercapnia reduces cAMP-stimulated anion secretion in Calu-3 cells, it was interesting to assess whether CO_{2} was modulating Cl^{-} or HCO_{3}^{-} secretion or indeed both. pH_{i} measurements were performed to indirectly measure HCO_{3}^{-} transport across the cells. At the apical membrane, we have previously shown that Calu-3 cells express the Cl^{-}/HCO_{3}^{-} anion exchanger pendrin, which mediates the majority of HCO_{3}^{-} efflux from the cell (Garnett et al., 2011). Pendrin activity was also shown to be regulated by CFTR. To measure CFTR-dependent pendrin activity, cells were stimulated with forskolin and pendrin activity assessed by Cl^{-} removal and readdition (Fig. 6A) (Garnett et al., 2011). In normocapnia, removal of apical Cl^{-} caused pH_{i} to increase by 0.61 ± 0.08 units (n=6), due to reversal of pendrin-mediated Cl^{-}/HCO_{3}^{-} exchange, whilst in hypercapnia this increase in pH_{i} was 0.64 ± 0.10 (p<0.05 vs. normocapnia; n=6 Fig. 6B). Furthermore, reintroduction of apical Cl^{-} caused pH_{i} to re-acidify at a rate of 0.49 ± 0.08 pH units min^{-1} in normocapnia and 0.45 ± 0.06 pH units min^{-1} in hypercapnia (p>0.05; n=6; Fig. 6C) which equated to a HCO_{3}^{-} efflux of 104 ± 21mM HCO_{3}^{-} min^{-1} and 127 ± 38mM HCO_{3}^{-} min^{-1}, respectively (p>0.05; n=6; Fig. 6D). It is important to note that in forskolin-stimulated conditions, the basolateral anion exchanger, AE2,
Acute hypercapnia does not alter cAMP-stimulated NBC activity in Calu-3 cells. To investigate HCO₃⁻ transport across the basolateral membrane, we measured the activity of NBC transporters which have been shown to mediate basolateral membrane HCO₃⁻ import in Calu-3 cells (Lee et al., 1998; Devor et al., 1999; Shan et al., 2012). NBC activity was monitored by measuring changes in pH, following the removal of basolateral Na⁺ (to inhibit NBC) and the readdition of basolateral Na⁺ (to re-activate NBC), as described by Yang et al. (2009), in the presence of EIPA to inhibit NHE activity. However, it was first necessary to determine whether NBC activity in Calu-3 cells was cAMP-dependent. Figures 7A and 7B show that both forskolin and adenosine stimulated a 2.3 ± 0.4 fold (n=3; p<0.05) and 2.5 ± 0.5 fold (n=3; p<0.05) increase, respectively, in NBC activity, under normocapnic conditions, indicating that NBC activity in Calu-3 cells is increased by cAMP.

The effect of acute hypercapnia on cAMP-regulated NBC activity was next assessed. Here, NBC activity was measured in normocapnic conditions (Fig. 7A) or after cells had been exposed to 20 mins of hypercapnia (Fig 7C). As summarised in Fig. 7D, forskolin stimulated an NBC-dependent HCO₃⁻ influx of 12.5 ± 1.8mM min⁻¹ (n=7) under normocapnia whilst in hypercapnia, forskolin-stimulated NBC-dependent HCO₃⁻ influx was 11.3 ± 1.7mM min⁻¹ (n=7; p<0.05 vs. normocapnia). These findings suggest that, like pendrin, acute hypercapnia does not affect cAMP-stimulated NBC activity and thus imply that CO₂-induced effects on cAMP-regulated anion transport were not due to changes in HCO₃⁻ secretion per se and suggested only Cl⁻ secretion was sensitive to elevated CO₂.

Hypercapnia reduces the volume of forskolin-stimulated fluid secretion in Calu-3 cells but has no effect on pH. We have previously shown that stimulation of Calu-3 cells with forskolin for 24 hours increased the secretion of a HCO₃⁻ rich fluid. Furthermore, based on pharmacological and genetic knock down experiments, we suggested that cAMP-stimulated liquid secretion was primarily regulated by CFTR, while HCO₃⁻ secretion was not directly via CFTR but through Cl⁻/HCO₃⁻ via pendrin (Garnett et al., 2011; Garnett et al., 2013). Given that it appears separate transporters were responsible for Cl⁻ and HCO₃⁻ secretion in Calu-3 cells, it was of interest to assess if hypercapnia impacted upon forskolin-stimulated ion and fluid secretion. Calu-3 cells were stimulated with forskolin in either 5% CO₂ (v/v) in air or 10% CO₂ (v/v) in air for 24 hours and the amount and pH of the secreted fluid analysed. Note that TEER was not significantly different between normocapnic controls (682 ± 28 Ω cm²; n=6) and cells incubated for 24 hours in hypercapnia (681 ± 6 Ω cm²; p>0.05 vs. control; n=6) suggesting that chronic hypercapnia did not alter tight junction properties of Calu-3 cells. In normocapnic conditions, unstimulated cells secreted 12 ± 4µl fluid over 24 hours (n=3) which was significantly enhanced 3.9 ± 0.2 fold to 49 ± 3µl by forskolin stimulation (p<0.01 vs. unstimulated cells; n=3; Fig. 8A). In hypercapnic conditions, unstimulated cells secreted 12 ± 1µl fluid over 24 hours which was almost identical to that seen in normocapnia (p>0.05; n=3). However, although forskolin increased fluid secretion to 32 ± 1µl over 24 hours (p<0.01; n=3; Fig. 8A), this 2.7 ± 0.1 fold increase in the volume of forskolin-stimulated fluid secretion was significantly lower than that observed in normocapnia (p<0.05 vs. normocapnia; n=3; Fig. 8A). This suggested chronic hypercapnia impaired cAMP-regulated CFTR-dependent Cl⁻ secretion in airway epithelia to reduce the osmotic driving force for fluid secretion. The pH of the secreted fluid was also measured. In normocapnia, the pH of secreted fluid increased from 7.52 ± 0.01 to 7.82 ± 0.06 (p<0.01; n=3) indicative of a greater [HCO₃⁻] in forskolin-stimulated fluid secretion. This pH increase of 0.31 ± 0.01 was not different to the pH increase of 0.30 ± 0.01 observed in hypercapnia (7.21 ± 0.04 to 7.51 ± 0.02; p>0.01 vs. unstimulated controls; p=0.05 vs. normocapnia; n=3; Fig. 8B) with the lower pH values observed due to acidosis induced by elevated CO₂. Using the Henderson-Hasselbalch equation to calculate [HCO₃⁻] revealed that the forskolin-stimulated fluid contained 61.6 ± 9.5mM HCO₃⁻ in normocapnia, which was not significantly different to the 58.2 ± 2.4mM HCO₃⁻ in the forskolin-stimulated fluid in hypercapnia (p>0.05; n=3). Together, these findings suggest that CFTR-dependent electrogenic Cl⁻ secretion is CO₂-sensitive, whilst pendrin-dependent HCO₃⁻ secretion is CO₂-insensitive, and supports the findings from Iₙc and pH measurements (Figs. 2, 6 and 7). In addition
since mucin secretion has been shown to be dependent on [HCO₃⁻] (Garcia et al., 2009; Chen et al., 2010; Gustafsson et al., 2012; Ridley et al., 2014), we also analysed the glycoprotein content of the secreted fluid by the PAS assay. In normocapnia, forskolin did not alter the amount of glycoproteins detected relative to unstimulated cells (18.5 ± 0.5µg/ml vs. 18.2 ± 1.0µg/ml respectively; p>0.05; n=3; Fig. 8C). Furthermore, hypercapnia had no effect on glycoprotein secretion from Calu-3 cells relative to normocapnia in either basal or forskolin-stimulated cells. Unstimulated cells secreted 19.2 ± 0.1µg/ml glycoprotein (p>0.05 vs. unstimulated cells in normocapnia; n=3) which was unchanged in response to forskolin stimulation (24.0 ± 4.0µg/ml; p>0.05 vs. unstimulated cells in hypercapnia; p>0.05 vs. stimulated cells in normocapnia; n=3; Fig. 8C). Therefore, hypercapnia modulated transporters involved in regulating the volume of secreted fluid but not those involved in mediating its composition.

Hypercapnia reduces forskolin-stimulated increases in Iₑ across primary human bronchial epithelial cells. To assess whether hypercapnia elicited similar effects in primary airway epithelia as it did in an airway epithelial cell line, Iₑ measurements were made on fully differentiated primary human bronchial epithelial cells (HBECs) grown under ALI. Figures 9A and 9B show representative experiments performed in conditions of normocapnia and hypercapnia, respectively. Hypercapnia had no effect on basal Iₑ, (basal Iₑ = 4.3 ± 1.1µA cm⁻² in normocapnia and 3.8 ± 0.5µA cm⁻² in acute hypercapnia; p>0.05 vs. normocapnia; n=6; Fig. 9C). However, it was found that the basal Iₑ was sensitive to apical amiloride (10µM) which reduced basal Iₑ by 5.0 ± 0.9µA cm⁻² in normocapnia (n=6) and 4.4 ± 0.6µA cm⁻² in hypercapnia (p<0.05 vs. normocapnia; n=6), suggesting ENaC activity was present in these cells. Stimulation of cells with forskolin in normocapnia induced a maximal increase in Iₑ of 13.9 ± 1.8µA cm⁻² (n=6) which was significantly reduced to 8.8 ± 1.3µA cm⁻² in cells that had been exposed to acute hypercapnia (p<0.05 vs. normocapnia; n=6; Fig. 9D). Furthermore, the rate of forskolin-stimulated Iₑ increase was also significantly reduced from 31.3 ± 4.4µA cm⁻² min⁻¹ (n=6) in normocapnia to 18.1 ± 2.6µA cm⁻² min⁻¹ in hypercapnia (p<0.05 vs. normocapnia; n=6; Fig. 9E). These data are consistent with the findings from Calu-3 cells and suggest that hypercapnia reduces cAMP-stimulated CFTR-dependent anion transport in primary human airway epithelial cells as well as in an airway epithelial cell line. When measuring the amount of CFTRinh-172-sensitive current, it was again found that there was a clear trend for this to be lower in acute hypercapnia, supporting the findings that CFTR activity was reduced by 10% CO₂. As shown in Fig. 9F, in normocapnia, forskolin-stimulated CFTRinh-172-sensitive current was 8.3 ± 1.6µA cm⁻² and was reduced in hypercapnia to 4.4 ± 0.9µA cm⁻² (n=6; p>0.05 vs. normocapnia; Fig. 9F).

Discussion

The ability of CO₂ to act as a cell signalling molecule is currently gaining substantial support within human physiology. Here we show, for the first time, that hypercapnia modulates cAMP-dependent signalling, as well as cAMP-dependent ion and fluid transport, in both a human airway epithelial cell line and also in primary human bronchial epithelial cells. We found that acute hypercapnia caused a significant reduction in forskolin stimulated [cAMP]ᵢ levels in Calu-3 cells – even in the presence of a PDE inhibitor - which was independent of CO₂-induced intracellular or extracellular acidosis (Fig. 1B). Interestingly, hypercapnia did not affect cAMP levels in cells stimulated with IBMX only (Fig. 1B) implying that the CO₂-induced attenuation of [cAMP], was not due to modulation of PDE activity consistent with our previous results (Townsend et al., 2009; Cook et al., 2012). The apparent lack of effect of hypercapnia in the absence of forskolin suggests that in order for hypercapnia to alter tmAC activity, the cyclase needs to be in an active state. Zhang et al. (1997) have described the presence of hydrophobic forskolin binding pockets on tmAC and forskolin binding at these sites induces a conformational change leading to dimerization of the two catalytic subunits of tmAC. Thus, it seems likely that CO₂ can only modulate tmAC activity when it is held within this “forskolin-bound” state. Similar conformational changes in tmAC are induced when free Gαᵣ bind to the enzyme, implying CO₂ modulates tmAC activity via the same mechanism when cells are stimulated with G-protein coupled receptor agonists such as adenosine (Tesmer et al., 1997).

The hypercapnic-induced reduction in forskolin-stimulated cAMP levels also had significant effects on forskolin-stimulated transepithelial ion transport in Calu-3 cells. In the presence of a basolateral to apical Cl⁻ gradient, 10% CO₂ caused a ~45% reduction in the rate of forskolin-stimulated increase in CFTRinh-172 and bumetanide-sensitive Iₑ (Fig. 2E). These findings imply that
CO₂-induced changes in [cAMP] secretion in Calu-3 cells. Hypercapnia also produced the same effect when cells were stimulated with the physiological cAMP agonist adenosine but did not alter IBMX-stimulated changes in Iₑₑ (Fig. 3). These findings indicated that CO₂-dependent reductions in [cAMP] were a result of modulations to tmAC-dependent cAMP production as opposed to PDE-dependent cAMP breakdown which supports previous findings from our laboratory (Townsend et al., 2009; Cook et al., 2012). We were also able to conclude that the modulations to cAMP-regulated anion transport in hypercapnia was not a result of the CO₂-induced intracellular acidosis as mimicking this acid load using sodium acetate did not alter forskolin-stimulated increases in Iₑₑ (Fig. 4).

Biotinylation experiments further showed that the effect of hypercapnia on Iₑₑ could not be explained by a reduction in surface levels of CFTR (Fig. 5). These findings support our hypothesis that in cAMP-stimulated conditions, the effects of CO₂ were due to modulation of [cAMP], as opposed to CO₂-dependent effects on pathways involved in regulating CFTR surface expression, for instance endocytosis. Furthermore, these findings are of particular relevance given that hypercapnia has been shown to modulate the surface expression of the Na⁺/K⁺-ATPase in mammalian alveolar epithelia (Briva et al., 2007), which therefore suggests that CO₂ only induces endocytosis of specific ion transporters. Acute hypercapnia also significantly lowered basal Iₑₑ in Calu-3 cells. Given that a large component of this basal Iₑₑ was sensitive to CFTRinh-172 suggests that hypercapnia also reduced the activity of CFTR under these conditions. However, because hypercapnia did not alter levels of [cAMP] under resting conditions (Fig. 1B), nor did hypercapnia alter surface CFTR expression (Fig. 5), indicates that the effect of high CO₂ on resting CFTR activity was independent of its effects on cAMP and not due to loss of CFTR at the plasma membrane. Therefore, why we observed a decrease in basal Iₑₑ in Calu-3 cells exposed to acute hypercapnia remains unclear but we cannot exclude the possibility that hypercapnia may have effects on basal [cAMP], which cannot be detected using our current method of quantification. It is important to note that whilst hypercapnia induces a reversible intracellular acidosis (Fig. 1A) and that CFTR has been shown to be pH-sensitive (Reddy et al., 1998; Chen et al., 2009; Melani et al., 2010), the 10% CO₂-induced acidosis of ~0.2 units is unlikely to significantly alter CFTR activity based on single channel recordings of CFTR expressed in mammalian cells (Chen et al., 2009) and measurements of CFTR-dependent Cl⁻ conductance made in human sweat ducts (Reddy et al., 1998). Furthermore, the fact that all measurements of cAMP-stimulated CFTR activity were made after cells had recovered pH₂ in response to CO₂-induced acidosis also strongly argues against any pH₂-dependent effects on CFTR activity in hypercapnia.

To identify the transport of which anion (Cl⁻ or HCO₃⁻) hypercapnia was modulating, intracellular pH measurements were performed to indirectly measure HCO₃⁻ transport in real time in polarised cultures of Calu-3 cells. Importantly, we showed that cAMP-stimulated, pendrin-dependent apical HCO₃⁻ secretion and cAMP-stimulated, NBC-dependent basolateral HCO₃⁻ influx were both insensitive to hypercapnia (Figs. 6 and 7), suggesting that hypercapnia did not alter HCO₃⁻ transport directly in Calu-3 cells. Thus the results from the Iₑₑ measurements suggested that the CO₂-induced reduction in electrogenic anion secretion was specifically due to a reduction in transepithelial Cl⁻ secretion. Thus, it appears that cAMP-regulated transporters have different sensitivities to CO₂-induced decreases in [cAMP] in Calu-3 cells. Although the reasons for this are unclear at the present time, it is known that CFTR exists in a microdomain at the apical membrane of airway epithelial cells, in which cAMP signalling is highly compartmentalized (Barnes et al., 2005; Penmatsa et al., 2010). A decrease in cAMP levels in such a compartmentalized microdomain would have more pronounced effects than in areas of the cell where cAMP signalling is less compartmentalized; for instance at the basolateral subcellular location. Similarly, apical and basolateral microdomains may possess distinct tmAC isoforms which could display differential sensitivities to raised CO₂.

We also observed similar results when investigating the effects of hypercapnia on cAMP-stimulated anion and fluid transport using a different approach. Incubating cells for 24 hours in hypercapnia enabled us to assess the effect of hypercapnia on the volume, as well as the composition of the secreted fluid (Fig. 8). We found that hypercapnia did not affect the amount of fluid secreted under basal conditions. This is consistent with results from Fig. 1B that demonstrated cAMP levels in non-stimulated Calu-3 cells were insensitive to hypercapnia. However, the fluid secretion data do contradict our Iₑₑ measurements in which CFTRinh-172-sensitive basal Iₑₑ was reduced in hypercapnia, suggesting that CFTR may be altered by hypercapnia through a cAMP-independent mechanism.
Nonetheless, hypercapnia caused a significant reduction in the amount of secreted fluid under forskolin-stimulated conditions (Fig. 8A). Given we have previously shown that the volume of forskolin-stimulated fluid secretion is predominantly mediated by electrogenic CFTR-dependent Cl– secretion, (31), strongly suggests that hypercapnia reduced fluid secretion via an effect on CFTR-dependent Cl– transport. This was likely due to the CO2-induced reduction in forskolin-stimulated cAMP levels (Fig. 1B). Although we demonstrated chronic hypercapnia did not affect the transepithelial resistance of Calu-3 monolayers, indicating paracellular ion and fluid transport was not altered by 10% CO2, one cannot rule out the possibility that hypercapnia may alter the water permeability of the epithelial monolayer which would be another interesting effect of elevated CO2.

However, unpublished findings from our laboratory have found that the osmolarity of secreted fluid in Calu-3 cells is unchanged in forskolin-stimulated cells compared to control cells. Thus, as we know forskolin to increase ion and fluid secretion in Calu-3 cells, these findings demonstrate changes in transepithelial ion secretion does not alter water permeability and thus is unlikely to contribute to the changes in fluid secretion observed in hypercapnia. Kim et al. (2014) also suggest water permeability is unchanged in Calu-3 cells even in conditions where ion secretion is stimulated. Interestingly, the [HCO3] of forskolin-stimulated fluid secretion was unaffected by chronic hypercapnia (Fig. 8B).

Garnett et al. (2011) demonstrated that the pH of forskolin-secreted fluid was predominately regulated by the Cl/HCO3 exchanger pendrin, and not directly by CFTR, since fluid pH was insensitive to GlyH-101 or genetic knockdown of CFTR, but was reduced by pendrin KD. Thus, our results demonstrate that CFTR and pendrin exhibit differential sensitivities to CO2. In addition, neither forskolin nor hypercapnia had any effect on the amount of glycoprotein detected in apical secretions from Calu-3 cells, suggesting that neither treatment modified mucus secretion. Kreda et al. (2007) demonstrated that secretion of mucus by Calu-3 cells, including MUC5AC, was a result of Ca2+-dependent exocytosis of mucin granules which likely explains why forskolin did not alter mucus secretion. Furthermore, these findings also imply that hypercapnia does not alter Ca2+-dependent mucin secretion and therefore only modulates cAMP-regulated responses.

Finally, the findings of acute hypercapnia on CFTR-dependent Ic in Calu-3 cells were also replicated in fully differentiated HBECs. In these cells 10% CO2 also significantly reduced cAMP-stimulated CFTR-dependent anion transport (Fig. 9). Although we did not measure [cAMP], in response to hypercapnia in HBECs, the ~42% decrease in the rate of forskolin-stimulated Ic increase in HBECs was comparable to the ~45% decrease observed in Calu-3 cells, and thus suggests CO2 elicited its effects via similar mechanisms in both cell types. However, one interesting difference was the fact that hypercapnia had no effect on basal Ic in HBECs where it did in Calu-3 monolayers (see Figs. 2C and 9C) suggesting that basal CFTR activity is less sensitive to CO2 in primary airway epithelia. However, given that basal Ic in Calu-3 cells was amiloride-insensitive (unpublished observations), as opposed to the large component of basal Ic in HBECs that was inhibited by amiloride, suggests different transporters regulate basal Ic in the two cell types and which likely explains the differences in response to hypercapnia. Furthermore, given there was no effect of CO2 on amiloride-sensitive Ic in HBECs suggested ENaC activity was insensitive to acute hypercapnia. This reinforces the findings that acute hypercapnia mediates specific effects on CFTR as opposed to other membrane ion transporters.

In summary, we have shown for the first time that acute hypercapnia reduced cAMP production as well as cAMP-stimulated, CFTR-dependent Cl–, but not HCO3–, secretion in human airway epithelia cells. We propose that CO2-induced reductions in cytosolic cAMP inhibit CFTR activity and thus CFTR-dependent Cl– secretion. However the lack of an effect on pendrin-dependent HCO3– secretion implies that there was sufficient residual CFTR activity to maintain Cl–/HCO3– exchange by pendrin, and thus efficient HCO3– secretion persisted. This is consistent with our previous results in which we showed significant pendrin-mediated anion exchange activity was still present in Calu-3 cells where CFTR levels were knocked down by ~75% (Garnett et al., 2011). However, dysregulation of CFTR-dependent Cl– and fluid secretion would be predicted to reduce airways hydration and compromise the innate defence mechanisms of the lungs (Pezzulo et al., 2012) predisposing the airways to bacterial colonization. These findings are of particular relevance to patients suffering from chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) or severe CF, in which bacterial infection is a major problem and hypercapnia is a complication. Thus, based on our findings, hypercapnia may be an additional contributing factor to airways
pathophysiology in these situations (Lourenco & Miranda, 1968; Holland et al., 2003; Sheikh et al., 2011). However, the effects of hypercapnia that we have reported should also be considered for those patients receiving treatment from Acute Respiratory Distress Syndrome (ARDS) who suffer from pulmonary edema due to increased permeability of the alveolar epithelium (Grommes & Soehnlein, 2011). These patients become hypercapnic as a consequence of their clinical treatment (Prin et al., 2002) and it has been postulated that it is the elevated CO₂ that provides the beneficial effects of the treatment. We suggest that a potential protective role of hypercapnia for ARDS patients could be in the reduction in the amount of cAMP-stimulated fluid secretion in the airways which would help minimize the extent of the edema without compromising the pH-dependent components of the airway innate defence mechanisms. Interestingly, our findings somewhat contradict those published by the Snzajder group who demonstrated that (i) hypercapnia reduced alveolar fluid reabsorption and thus increased pulmonary edema in rat alveolar cells (Briva et al., 2007; Vadasz et al., 2008) (ii) high CO₂ increased apical [cAMP], in both A549 cells and rat alveolar type II cells (Lecuona et al., 2013). The findings reported here highlight potential differences in CO₂ signalling between rat and humans as well as suggesting that secretory cells of the conducting airways respond differently to hypercapnia compared to absorptive cells of the respiratory airways. Several studies have also implicated CO₂ as an anti-inflammatory agent (Laffey et al., 2000; Sinclair et al., 2002; De Smet et al., 2007; Contreras et al., 2012; Oliver et al., 2012) whilst hypercapnia has also been shown to attenuate ventilator-induced lung injury in mice (Otulakowski et al., 2014). Our findings may suggest another possible protective role of hypercapnia in ARDS patients which would complement the other reported benefits of hypercapnia.
References


Additional Information

Competing Interests

None declared

Author Contributions

M.J.T., M.J.C. and M.A.G. conceived and designed the experiments.
M.J.T., V.S., W.P., S.I. and B.V. conducted experiments and collected data.
M.J.T., V.S. and W.P. performed data analysis.
J.P.G. and C.W. provided resources.
M.J.T., C.W., R.T., M.J.C. and M.A.G. drafted the article or revised it critically for important intellectual content.

Funding

This work was supported by an MRC Studentship awarded to M.J.T.; a BBSRC studentship to W.P. and an overseas studentship to S.H.I. funded by the Higher Committee for Education Development (HCED), Iraq. Additional funding was also provided by the Cystic Fibrosis Trust (Grant SRC003).

Acknowledgements

The authors acknowledge the technical expertise of Yishan Luo in assisting with cell surface biotinylation experiments.
Figure 1. Acute hypercapnia attenuates forskolin-stimulated cAMP levels in Calu-3 cells independent of changes in intracellular pH. (A) shows the effect of hypercapnia (10% CO₂) on the pH_i of Calu-3 cells and demonstrates cells recovered pH_i from CO₂-induced acidosis after ~20 mins. (B) shows the effect of acute hypercapnia on intracellular cAMP in which cells were incubated for 20 mins in either 5% CO₂ (v/v) in air or 10% CO₂ (v/v) in air before being stimulated with either IBMX (1 mM) or forskolin (5 μM) + IBMX (1 mM) for a further 10 mins. Intracellular cAMP levels were determined by measuring the amount of [³H]-cAMP in each sample. *** = significant effect of forskolin (p<0.001; * = p<0.05); † = significant effect of hypercapnia (p<0.05). Data represents mean ± S.E.M.; n = 6 for each.

Figure 2. Forskolin-stimulated transepithelial anion secretion is reduced in conditions of acute hypercapnia in Calu-3 cells. Calu-3 cells were grown on permeable Snapwell supports and I_{sc} was measured using an Ussing chamber. (A) shows a representative I_{sc} recording of a control experiment in which cells were exposed to 5% (v/v) CO₂/95% (v/v) O₂ and (B) shows a representative recording in which cells were pre-exposed to 10% (v/v) CO₂/90% (v/v) O₂ for 20 mins prior to being studied. Apical [Cl⁻] was reduced to 40mM and cells were stimulated with forskolin (Fsk; 5μM) before addition of apical CFTR\textsubscript{inh}-172 (20μM) and basolateral bumetanide (Bumet; 25μM) as indicated. The basal I_{sc} (C), the maximal forskolin-stimulated increase in I_{sc} (D), the rate of increase in forskolin-stimulated I_{sc} (E) and the amount of forskolin-stimulated current that was inhibited by CFTR\textsubscript{inh}-172 (F) are displayed. ** = significant effect of hypercapnia (p<0.01). Data represents mean ± S.E.M.; n=10 for normocapnia and n=8 for hypercapnia.

Figure 3. Acute hypercapnia reduces adenosine but not IBMX-stimulated transepithelial anion secretion in Calu-3 cells. Calu-3 cells were grown on permeable Snapwell supports and I_{sc} was measured using an Ussing chamber. For control experiments, cells were gassed with 5% (v/v) CO₂/95% (v/v) O₂ whilst hypercapnia was induced by pre-exposing cells to 10% (v/v) CO₂/90% (v/v) O₂ for 20 mins prior to being studied. Apical [Cl⁻] was reduced to 40mM and cells were stimulated with either adenosine (10μM) or IBMX (1 mM) before addition of apical CFTR\textsubscript{inh}-172 (20μM) and basolateral bumetanide (25μM). (A) shows a representative experiment in which cells were incubated for 20 mins in either 80mM mannitol or 40mM sodium acetate and exposed to 40mM sodium acetate and CO₂/90% (v/v) O₂ for 20 minutes prior to addition of forskolin (Fsk; 5μM), apical CFTR\textsubscript{inh}-172 (20μM) and basolateral bumetanide (Bumet; 25μM) as indicated. (E) and (F) display the rate of increase in adenosine-stimulated I_{sc}. * = significant effect of hypercapnia (p<0.05). Data represents mean ± S.E.M.; n=5 for normocapnia and n=3 for hypercapnia. (C) and (D) display the rate of increase in IBMX-stimulated I_{sc}. Data represents mean ± S.E.M.; n=4 for normocapnia and n=3 for hypercapnia.

Figure 4. The effect of hypercapnia on cAMP-dependent transepithelial anion secretion is independent of CO₂-induced intracellular acidosis. (A) summarizes the magnitude of the intracellular acidosis resulting from either 10% CO₂ or sodium acetate. Data represents mean ± S.E.M., n=60 for 10% CO₂; n= 6 for sodium acetate. (C) and (D) show representative I_{sc} measurements in which cells were exposed to 80mM mannitol or 40mM sodium acetate respectively for 20 minutes prior to addition of forskolin (Fsk; 5μM), apical CFTR\textsubscript{inh}-172 (20μM) and basolateral bumetanide (Bumet; 25μM) as indicated. (E) and (F) summarize the effect of sodium acetate on the magnitude and the rate of the forskolin-stimulated increase in I_{sc} respectively. Data represents mean ± S.E.M., n=5 for each.

Figure 5. Cell surface expression of CFTR is unaffected by acute hypercapnia. Calu-3 cells were grown on permeable transwell supports and membrane expression of CFTR was assessed using a biotinylation assay. (A) displays an example blot of whole cell CFTR expression under 5% CO₂ and 10% CO₂ and the relative expression of whole cell CFTR when normalized to expression of whole cell α-tubulin. Data represents mean ± S.E.M.; n = 5. (B) displays an example blot of biotinylated CFTR expression, used as a marker of surface expression, under 5% CO₂ and 10% CO₂ and the relative expression of biotinylated CFTR when normalized to expression of biotinylated α-tubulin. Data represents mean ± S.E.M.; n=4.
Figure 6. CFTR-regulated, pendrin-dependent apical HCO$_3^-$ efflux is unaffected by hypercapnia. (A) shows a representative pH$_i$ experiment in which the effect of acute hypercapnia on 5μM forskolin-stimulated, CFTR-regulated apical HCO$_3^-$ transport was assessed by removal and subsequent readdition of apical Cl$. The delta pH in response to removal of Cl$ is shown in (B). The rate of reacidification and HCO$_3^-$ flux resulting from readdition of apical Cl$ are shown in (C) and (D) respectively. Data represents mean ± S.E.M.; $n=6$ for each.

Figure 7. Hypercapnia does not alter cAMP-stimulated NBC activity in Calu-3 cells. (A) shows a representative pH$_i$ experiment in which NBC activity was assessed under basal and forskolin-stimulated conditions in 5% CO$_2$. EIPA (3μM) was present to inhibit the NHE. (B) shows the effect of the cAMP agonists forskolin (5μM) and adenosine (10μM) on NBC-dependent HCO$_3^-$ influx. * = significant effect of agonist stimulation; (p<0.05). Data represents mean ± S.E.M.; $n=3$ for each. (C) shows a representative pH$_i$ experiments in which forskolin-stimulated NBC activity was assessed in conditions of acute hypercapnia. EIPA (3μM) was present to inhibit the NHE. (E) displays the effect of hypercapnia on forskolin-stimulated NBC activity. Data represents mean ± S.E.M., $n=7$ for each.

Figure 8. Hypercapnia reduces the volume of forskolin-stimulated fluid secretion in Calu-3 cells. Cells were stimulated with forskolin (Fsk; 5μM) and incubated for 24 hours in either 5% CO$_2$ (v/v) in air or 10% CO$_2$ (v/v) in air in high Cl$-Krebs$ solution at 37°C. (A) shows the effect of chronic hypercapnia on the volume of fluid secreted over 24 hours. ** = significant effect of forskolin stimulation compared to unstimulated control cells (p<0.01; *** = p<0.001); † = significant effect of 10% CO$_2$ (p<0.05). Data represents mean ± S.E.M.; $n=3$ for each. (B) displays the increase in pH of forskolin-stimulated secreted fluid relative to unstimulated control cells. Data represents mean ± S.E.M.; $n=3$ for each. (C) displays the effects of forskolin and hypercapnia on the amount of glycoprotein present in the secreted fluid, quantified by the PAS assay. Data represents mean ± S.E.M.; $n=3$ for each.

Figure 9. Forskolin-stimulated transepithelial anion secretion is reduced in conditions of acute hypercapnia in primary human bronchial epithelial cells. Primary human bronchial epithelial cells were grown on collagen coated permeable Snapwell supports and allowed to differentiate at a ALI for 30-35 days before $I_{sc}$ was measured using an Ussing chamber. (A) shows a representative $I_{sc}$ recording of a control experiment in which cells were exposed to 5% (v/v) CO$_2$/95% (v/v) O$_2$ and (B) shows a representative recording in which cells were pre-exposed to 10% (v/v) CO$_2$/90% (v/v) O$_2$ for 20 mins prior to being studied. Apical [Cl$^-$] and basolateral [Cl$^-$] were both 124mM for these experiments. Cells were treated with apical amiloride (Amil; 10μM) stimulated with forskolin (Fsk; 10μM) before addition of apical CFTRinh-172 (20μM) as indicated. The basal $I_{sc}$ (C), the maximal forskolin-stimulated increase in $I_{sc}$ (D), the rate of increase in forskolin-stimulated $I_{sc}$ (E) and the amount of forskolin-stimulated current that was inhibited by CFTRinh-172 (F) are displayed. * = significant effect of hypercapnia (p<0.05). Data represents mean ± S.E.M.; $n = 6$ for each.
Figure 1

A

B

5% CO₂

10% CO₂

pH

8.0

7.8

7.6

7.4

7.2

1005

Ratio

[3H]-cAMP/Total [3H]

IBMX

Fsk + IBMX

0.0

0.2

0.4

0.6

0.8

1.0

1.2

1.4

1.6

5% CO₂

10% CO₂

***

†

*
Figure 2

A  
40mM Cl (ap)  Fsk  CFTR
40mM Cl (ap)  Fsk  CFTR

B  
40mM Cl (ap)  Fsk  CFTR

C  
\[ I_{sc} \text{ (µA cm}^2\text{)} \]

D  
\[ \text{Delta } I_{sc} \text{ (µA cm}^2\text{)} \]

E  
\[ \text{Rate of fsk-induced } I_{sc} \text{ increase (µA cm}^2\text{ min}^{-1}\text{)} \]

F  
\[ \text{CFTR}_{inh} \text{ inhibitable } I_{sc} \text{ (µA cm}^2\text{)} \]
Figure 3

A) Delta $I_{sc}$ (µA cm$^{-2}$)  

B) Rate of ado-induced $I_{sc}$ increase (µA cm$^{-2}$ min$^{-1}$)  

C) Delta $I_{sc}$ (µA cm$^{-2}$)  

D) Rate of IBMX-induced $I_{sc}$ increase (µA cm$^{-2}$ min$^{-1}$)
Figure 4

A

+ 40mM Na Acetate

7.8
7.6
7.4
7.2
7.0
pH

B

[Graph showing Delta pH values for 10% CO2 and Na Acetate conditions]

C

[Graph showing current (Isc) changes with Fsk, CFTRinh, and Bumet]

D

[Graph showing current (Isc) changes with Fsk, CFTRinh, and Bumet]

E

[Bar graph showing Delta Isc comparisons between Mannitol and NaAcetate]

F

[Bar graph showing the rate of fsk-induced Isc increase between Mannitol and NaAcetate]
Figure 5

A

5% 10%

CFTR

α-Tub

150kD

50kD

Expression of whole cell CFTR (Normalized to expression of α-tubulin)

B

5% 10%

CFTR

α-Tub

150kD

50kD

Expression of Biotinylated CFTR (Normalized to expression of α-tubulin)
Figure 6

A

\[ \text{pH} \]

\[ \begin{array}{c}
\text{5\% CO}_2 \\
\text{Fsk} \\
\text{0Cl} \\
\text{10\% CO}_2 \\
\text{Fsk} \\
\end{array} \]

250s

B

Delta pH

5\% 10\%

C

Rate of reacidification (pH units min\(^{-1}\))

5\% 10\%

D

\( \text{HCO}_3^- \text{ efflux (nM min}^{-1}\)

5\% 10\%
Figure 7

(A) 5% CO₂

(B) NBC-dependent HCO₃⁻ influx (μM/min⁻¹)

(Fsk) Basal

(Ado) Stimulated

(C) 5% CO₂ 10% CO₂

(D) Forskolin-stimulated, NBC-dependent HCO₃⁻ influx (μM/min⁻¹)

5% 10%
Figure 8

A. Volume of fluid secreted per transwell (µl)
B. Increase in pH of secreted fluid
C. Glycoprotein concentration in secreted fluid (µg/ml)
Figure 9

(A) Graph showing the change in $I_{sc}$ (µA cm$^{-2}$) with time (200s) following the application of Amiloride, Fsk, and CFTRinh-172.

(B) Graph showing the change in $I_{sc}$ (µA cm$^{-2}$) with time (200s) following the application of Amiloride, Fsk, and CFTRinh-172.

(C) Bar graph showing $I_{sc}$ (µA cm$^{-2}$) at 5% and 10%.

(D) Bar graph showing the delta $I_{sc}$ (µA cm$^{-2}$) at 5% and 10%.

(E) Bar graph showing the rate of Fsk-induced $I_{sc}$ increase (µA cm$^{-2}$ min$^{-1}$) at 5% and 10%.

(F) Bar graph showing the inhibitable $I_{sc}$ at 5% and 10%.