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

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design; Provision of study materials or patients; Manuscript Writing; Final approval of manuscript (required)

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

Mark J. Turner[1,5], Vinciane Saint-Criq[1],Waseema Patel[1], Salam H. Ibrahim[1], Bernard Verdon[1], Christopher Ward[2], James P. Garnett[1], Robert Tarran[3] Martin J. Cann[4] and Michael A. Gray[1]

[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.

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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 40mmHg 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, CO_2 is used to synthesize sugars during photosynthesis whilst in animals, although CO_2 is a waste product of cellular respiration, it also has an important roles in maintaining plasma pH *via* its buffering effect on HCO_3^- (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 CO_2 in arterial blood (hypercapnia) is associated with lung disease in humans (Lourenco & 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 CO_2 can act as a *bona fide* cell signalling molecule, and that changes in CO_2 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^+/HCO_3^- cotransporter (NBC) (Adijanto *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 CO_2 on membrane transporters has been shown to involve different mechanisms. For instance, CO_2-dependent downregulation of Na^+/K^+-ATPase activity specifically involves the endocytosis of the α subunit of the Na^+/K^+-ATPase, demonstrating that CO_2 can alter surface expression of ion transporters (Briva *et al*., 2007). Alternatively, CO_2 directly modulates connexin 26 *via* carbamylation, a post-translational modification whereby a covalent bond forms between the carbon in CO_2 and a primary amine group of the target protein (Meigh *et al*., 2013). In addition, CO_2 also has reported effects on key cell second messengers involved in membrane transporter regulation; specifically cyclic AMP and Ca^{2+} (Cann *et al*., 2003; Cann, 2004). cAMP is synthesized from ATP, a reaction catalysed by adenylyl cyclase, of which there exists both membrane-bound transmembrane 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% CO_2 compared to those exposed to 0.03% CO_2, demonstrating that tmAC is sensitive to changes in CO_2 (Townsend *et al*., 2009). This study also showed that tmAC was sensitive to CO_2 but not HCO_3^- *in vivo* and *in vitro*, supporting previous findings that first proposed tmAC activity was only sensitive to CO_2 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% CO_2 caused a significant reduction in both forskolin and parathyroid hormone-stimulated increases in intracellular cAMP ($[cAMP]_i$) compared to levels measured under normocapnic conditions of 5% CO_2 (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 CO_2 on cAMP was dependent on an IP_3-dependent release of Ca^{2+} which, in turn, led to an inhibition in tmAC activity, thereby demonstrating that CO_2 affected Ca^{2+} as well as cAMP signalling. These data supported earlier studies that demonstrated CO_2 modulated Ca^{2+} 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 secretions as well as SMG-derived secretory cell lines, such as Calu-3, have found that elevations in intracellular cAMP stimulate CFTR-dependent Cl^-, HCO_3^- 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 HCO_3^- secreting epithelia underlies the hereditary disease Cystic Fibrosis (CF) and airways dehydration and impaired ASL alkalinisation 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 HCO_3^- 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 HCO_3^- efflux *via* pendrin, and NBC-dependent HCO_3^- import were unaffected by hypercapnia. Furthermore, hypercapnia also reduced the volume of cAMP-stimulated fluid secretion without affecting the HCO_3^- content of the fluid, implying Cl^- secretion and HCO_3^- secretion have differential sensitivities to hypercapnia. Hypercapnia also reduced cAMP-stimulated anion secretion in primary human bronchial epithelial layers, indicating this effect of CO_2 would be predicted to occur *in vivo*. Our results therefore demonstrate that CO_2 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, 100Uml^{-1} penicillin and 100µgml^{-1} streptomycin. Cells were incubated at 37˚C in humidified air containing 5% (v/v) CO_2 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 cm^{-2} 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 surveilance bronchoscopy as previously described (Forrest *et al*., 2005). These were grown in a CO_2 incubator (37C; 5% CO_2) to 90% confluence using Bronchial Epithelial Growth Medium with supplements (BEGM, Lonza) in T_{25} 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 $AgCl_2$ 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) CO_2/95% (v/v) O_2 for control conditions or 10% (v/v) CO_2/90% (v/v) O_2 to induce hypercapnia. To measure the short circuit current (I_{sc}), 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 (V_{te}) to 0mV which was recorded as the I_{sc} using Scope 3 software (AD Instruments). To monitor transepithelial resistance (R_{te}), 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 fluor inverted microscope and perfused with a modified Krebs solution gassed with either 5% (v/v) CO_2/95% (v/v) or O_2 10% (v/v) CO_2/90% (v/v) O_2. 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 (pH_i) was measured using a Life Sciences Microfluorimeter System in which cells were alternatively excited at 490nm and 440nm

wavelengths every 1.024 s with emitted light collected at 510nm. The ratio of 490nm emission to 440nm 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 10μM nigericin, set to a desired pH, ranging from 6.6 to 8.4. Total buffering capacity (β_{tot}) was calculated by addition of the intrinsic buffering capacity (β_i) to the buffering capacity of the CO_2-HCO_3^- buffer system (βHCO_3^-) in which β_i was calculated using the NH_4^+ technique as described by Roos and Boron (1981). For analysis of pH_i measurements, delta pH_i (ΔpH_i) was determined by calculating the mean pH_i over 60 s resulting from treatment. Rate of pH_i change ($\Delta pH_i/\Delta t$) 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 $\Delta pH_i/\Delta t$ by β_{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} [^{3}H]-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 humidified 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 supernatent 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-α 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 % CO_2, had the pH assessed using a MiniTrode lab pH electrode (Hamilton, Reno, USA). This enabled the HCO_3^- concentration of the secreted fluid to be calculated using the Henderson-Hasselbalch equation, where; $pH = pK_a + \log_{10} ([HCO_3^-] / (0.03 \times pCO_2))$ where $pK_a = 6.1$ (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 added to 7% 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 $CFTR_{inh}$ 172 (Calbiochem, Watford, UK). All gas cylinders were purchased from BOC and consisted of the following mixtures: 5% CO_2/95% O_2 and 10% CO_2/90% O_2. NaHEPES solution consisted of (in mM) 130 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 NaHEPES and 10 D-Glucose, pH 7.4 at 37°C. High Cl^- Krebs solution consisted of (in mM) 25 $NaHCO_3$, 115 NaCl, 5 KCl, 1 $CaCl_2$ 1 $MgCl_2$ and 10 D-Glucose (pH 7.4). For high Cl^-, Na^+ free solutions, $NaHCO_3$ was replaced with choline bicarbonate and NaCl was replaced with NMDG-Cl. Zero Cl^- Krebs solution consisted of (in mM) 25 $NaHCO_3$, 115 NaGluconate, 2.5 K_2SO_4, 1 CaGluconate, 1 MgGluconate and 10 D-Glucose. Intracellular pH_i calibration solutions consisted of (in mM) 5 NaCl, 130 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 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 were set to desired pH by using 1M HCl or 1M NaOH. Solutions used to determine intracellular buffering capacity consisted of (in mM) 4.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 BaCl, 10 HEPES, 10 D-Glucose as well as varying concentrations of NH_4Cl/NMDG-Cl, ranging from 0 NH_4Cl/145 NMDG-Cl to 30 NH_4Cl/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 pH_i of Calu-3 cells since it is well known that raising CO_2 generally induces cytosolic acidification. Cells were first perfused with Krebs solution gassed with 5% (v/v) CO_2 to maintain cells in a normocapnic environment. Perfusing cells with 10% (v/v) CO_2, caused pH_i 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) CO_2 (Fig. 1A). We therefore chose 20 mins as an appropriate time to study the effects of acute hypercapnia as cells would have recovered their pH_i. Exposure of cells to 10% (v/v) CO_2 for 20 mins did not alter the integrity of the epithelial monolayer as assessed by recording TEER. In normocapnia, TEER was 671 ± 42Ω cm^{-2} (*n*=3) and 600 ± 42Ω cm^{-2} in monolayers of Calu-3 cells exposed to acute hypercapnia ($p>0.05$ *vs*. normocapnia; *n*=3). For all experiments, $[HCO_3^-]$ 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 CO_2-dependent effects on

tmAC as opposed to effects of HCO_3^- on sAC – an enzyme shown to be sensitive to HCO_3^- (Chen *et al*., 2000) In addition, given the scope of our work was to investigate the effect of raised CO_2 on bicarbonate secretion, changing [HCO_3^-] in hypercapnia would be predicted to compromise these studies.

As we have previously shown cAMP signalling was sensitive to changes in CO_2 (Townsend *et al*., 2009; Cook *et al*., 2012), intracellular cAMP levels ($[cAMP]_i$) were measured in conditions of normocapnia and after 20 mins exposure to hypercapnia, with the incubation media buffered to pH 7.4 in each condition to control for differences in extracellular pH (pH_e). In the presence of the non-specific phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX), there was no effect of hypercapnia on $[cAMP]_i$ (Fig. 1B). Stimulation of cells with the cAMP elevating agonist forskolin (added *after* 20 mins exposure to 5 or 10% CO_2 to allow for pH_i recovery) produced a 3.3 ± 0.5 fold increase in $[cAMP]_i$ in normocapnia ($p<0.001$; $n=6$; Fig. 1B) but this was significantly reduced to a 2.3 ± 0.4 fold increase in $[cAMP]_i$ in cells exposed to acute hypercapnia ($p<0.05$ *vs*. normocapnia; $n=6$; Fig. 1B). When the cAMP levels produced in IBMX-stimulated cells were subtracted from the cAMP levels measured in the presence of forskolin + IBMX, acute hypercapnia induced a 48 ± 4% reduction in $[cAMP]_i$. These results demonstrate that cAMP signalling in Calu-3 cells is responsive to elevated CO_2, through a mechanism that is independent of changes in pH_e and not due to the CO_2-induced intracellular acidosis.

Forskolin-stimulated transepithelial anion secretion is reduced in conditions of acute hypercapnia in Calu-3 cells. To assess whether the CO_2-induced reductions in forskolin-stimulated $[cAMP]_i$ modulated cAMP-regulated transepithelial ion transport, I_{sc} measurements were made in monolayers of Calu-3 cells. The I_{sc} is the current required to clamp the transepithelial voltage difference (V_{te}) to 0mV. In Calu-3 monolayers, the magnitude of the V_{te} 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 I_{sc} reflect changes in anion secretion. Figure 2A shows a representative recording of I_{sc} 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 I_{sc} of 5.2 ± 0.4μA and further investigations showed that this basal I_{sc} 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 $CFTR_{inh}$-172 (20μM) reduced basal I_{sc} by 48.5 ± 4.2% ($p<0.01$; $n=3$), indicating that the majority of basal I_{sc} was mediated by CFTR. Interestingly, in cells exposed to 20 mins hypercapnia (Fig. 2B), the basal I_{sc} 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 I_{sc} 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 I_{sc} was blocked by a combination of apical $CFTR_{inh}$-172 (20μM) and basolateral bumetanide (25μM), and both the magnitude and rate of I_{sc} increase were significantly reduced by 61.8 ± 16.0% and 73.4 ± 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 I_{sc}, consistent with previous studies (Welsh & Smith, 2001; Kreda *et al*., 2007; Shan *et al*., 2012). The maximal forskolin-stimulated increase in I_{sc} (ΔI_{sc}) 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 I_{sc} 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 I_{sc} that was sensitive to $CFTR_{inh}$-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 $[cAMP]_i$ 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 $[cAMP]_i$ 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 $[cAMP]_i$ was insensitive to CO_2, and suggest hypercapnia-induced changes in $[cAMP]_i$ 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 (see 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 the sodium acetate containing 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 $[cAMP]_i$ 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, was

almost completely inhibited, both in normocapnia (96.9 ± 1.9% inhibition; *n*=4) and hypercapnia (93.8 ± 4.3% inhibition; *n*=4) which is consistent with findings previously published by our laboratory (Garnett *et al*., 2011). Thus, AE2-dependent HCO_3^- transport can be eliminated from having any effect on these measurements. Therefore, these data show that apical CFTR-dependent anion exchange activity was unaffected by acute hypercapnia and suggested that HCO_3^- transport across the apical membrane was insensitive to changes in CO_2.

Acute hypercapnia does not alter cAMP-stimulated NBC activity in Calu-3 cells. To investigate HCO_3^- transport across the basolateral membrane, we measured the activity of NBC transporters which have been shown to mediate basolateral membrane HCO_3^- 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_i 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 HCO_3^- influx of 12.5 ± 1.8mM min^{-1} (*n*=7) under normocapnia whilst in hypercapnia, forskolin-stimulated NBC-dependent HCO_3^- 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 CO_2-induced effects on cAMP-regulated anion transport were not due to changes in HCO_3^- secretion *per se* and suggested only Cl^- secretion was sensitive to elevated CO_2.

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 HCO_3^- rich fluid. Furthermore, based on pharmacological and genetic knock down experiments, we suggested that cAMP-stimulated liquid secretion was primarily regulated by CFTR, while HCO_3^- secretion was not directly *via* CFTR but through Cl^-/HCO_3^- *via* pendrin (Garnett *et al*., 2011; Garnett *et al*., 2013). Given that it appears separate transporters were responsible for Cl^- and HCO_3^- 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% CO_2 (v/v) in air or 10% CO_2 (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 cm^{-2}; *n*=6) and cells incubated for 24 hours in hypercapnia (681 ± 6 Ω cm^{-2}; 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 $[HCO_3^-]$ 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 CO_2. Using the Henderson-Hasselbalch equation to calculate $[HCO_3^-]$ revealed that the forskolin-stimulated fluid contained 61.6 ± 9.5mM HCO_3^- in normocapnia, which was not significantly different to the 58.2 ± 2.4mM HCO_3^- in the forskolin-stimulated fluid in hypercapnia (p>0.05; *n*=3). Together, these findings suggest that CFTR-dependent electrogenic Cl^- secretion is CO_2-sensitive, whilst pendrin-dependent HCO_3^- secretion is CO_2-insenstive, and supports the findings from I_{sc} and pH_i measurements (Figs. 2,6 and 7). In addition

since mucin secretion has been shown to be dependent on [HCO_3^-] (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 I_{sc} 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, I_{sc} 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, resepectively. Hypercapnia had no effect on basal I_{sc}, (basal I_{sc} = 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 I_{sc} was sensitive to apical amiloride (10μM) which reduced basal I_{sc} 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 I_{sc} 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 I_{sc} 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 epithelia cell line. When measuring the amount of $CFTR_{inh}$-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% CO_2. As shown in Fig. 9F, in normocapnia, forskolin-stimulated $CFTR_{inh}$-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 CO_2 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 CO_2-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 CO_2-induced attenuation of $[cAMP]_i$ 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 CO_2 can only modulate tmAC activity when it is held within this "forskolin-bound" state. Similar conformational changes in tmAC are induced when free $G_{\alpha s}$ bind to the enzyme, implying CO_2 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% CO_2 caused a ~45% reduction in the rate of forskolin-stimulated increase in $CFTR_{inh}$-172 and bumetanide-sensitive I_{sc} (Fig. 2E). These findings imply that

CO_2-induced changes in $[cAMP]_i$ were sufficient to reduce CFTR-dependent electrogenic anion 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 I_{sc} (Fig. 3). These findings indicated that CO_2-dependent reductions in $[cAMP]_i$ 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 CO_2-induced intracellular acidosis as mimicking this acid load using sodium acetate did not alter forskolin-stimulated increases in I_{sc} (Fig. 4).

Biotinylation experiments further showed that the effect of hypercapnia on I_{sc} 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 CO_2 were due to modulation of $[cAMP]_i$ as opposed to CO_2-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 CO_2 only induces endocytosis of specific ion transporters. Acute hypercapnia also significantly lowered basal I_{sc} in Calu-3 cells. Given that a large component of this basal I_{sc} was sensitive to $CFTR_{inh}$-172 suggests that hypercapnia also reduced the activity of CFTR under these conditions. However, because hypercapnia did not alter levels of $[cAMP]_i$ under resting conditions (Fig. 1B), nor did hypercapnia alter surface CFTR expression (Fig. 5), indicates that the effect of high CO_2 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 I_{sc} in Calu-3 cells exposed to acute hypercapnia remains unclear but we cannot exclude the possibility that hypercapnia may have effects on basal $[cAMP]_i$ 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% CO_2-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 pH_i in response to CO_2-induced acidosis also strongly argues against any pH_i-dependent effects on CFTR activity in hypercapnia.

To identify the transport of which anion (Cl^- or HCO_3^-) hypercapnia was modulating, intracellular pH measurements were performed to indirectly measure HCO_3^- transport in real time in polarised cultures of Calu-3 cells. Importantly, we showed that cAMP-stimulated, pendrin-dependent apical HCO_3^- secretion and cAMP-stimulated, NBC-dependent basolateral HCO_3^- influx were both insensitive to hypercapnia (Figs. 6 and 7), suggesting that hypercapnia did not alter HCO_3^- transport directly in Calu-3 cells. Thus the results from the I_{sc} measurements suggested that the CO_2-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 CO_2-induced decreases in $[cAMP]_i$ 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 CO_2.

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 I_{sc} measurements in which $CFTR_{inh}$-172-sensitive basal I_{sc} 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 CO_2-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% CO_2, 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 CO_2. 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 $[HCO_3^-]$ 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^-/HCO_3^- 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 CO_2. 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 mucins by Calu-3 cells, including MUC5AC, was a result of Ca^{2+}-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 Ca^{2+}-dependent mucin secretion and therefore only modulates cAMP-regulated responses.

Finally, the findings of acute hypercapnia on CFTR-dependent I_{sc} in Calu-3 cells were also replicated in fully differentiated HBECs. In these cells 10% CO_2 also significantly reduced cAMP-stimulated CFTR-dependent anion transport (Fig. 9). Although we did not measure $[cAMP]_i$ in response to hypercapnia in HBECs, the ~42% decrease in the rate of forskolin-stimulated I_{sc} increase in HBECs was comparable to the ~45% decrease observed in Calu-3 cells, and thus suggests CO_2 elicited its effects *via* similar mechanisms in both cell types. However, one interesting difference was the fact that hypercapnia had no effect on basal I_{sc} in HBECs where it did in Calu-3 monolayers (see Figs. 2C and 9C) suggesting that basal CFTR activity is less sensitive to CO_2 in primary airway epithelia. However, given that basal I_{sc} in Calu-3 cells was amiloride-insensitive (unpublished observations), as opposed to the large component of basal I_{sc} in HBECs that was inhibited by amiloride, suggests different transporters regulate basal I_{sc} in the two cell types and which likely explains the differences in response to hypercapnia. Furthermore, given there was no effect of CO_2 on amiloride-sensitive I_{sc} 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 HCO_3^-, secretion in human airway epithelia cells. We propose that CO_2-induced reductions in cytosolic cAMP inhibit CFTR activity and thus CFTR-dependent Cl^- secretion. However the lack of an effect on pendrin-dependent HCO_3^- secretion implies that there was sufficient residual CFTR activity to maintain Cl^-/HCO_3^- exchange by pendrin, and thus efficient HCO_3^- 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 CO_2 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 contradicts 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 CO_2 increased apical $[cAMP]_i$ in both A549 cells and rat alveolar type II cells (Lecuona *et al*., 2013). The findings reported here highlight potential differences in CO_2 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 CO_2 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.

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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.

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Figure Legends

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% CO_2) on the pH_i of Calu-3 cells and demonstrates cells recovered pH_i from CO_2-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% CO_2 (v/v) in air or 10% CO_2 (v/v) in air before being stimulated with either IBMX (1mM) or forskolin (5μM) + IBMX (1mM) for a further 10 mins. Intracellular cAMP levels were determined by measuring the amount of [^{3}H]-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) 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^-] was reduced to 40mM and cells were stimulated with forskolin (Fsk; 5μM) before addition of apical $CFTR_{inh}$-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 inhibitied by $CFTR_{inh}$-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) CO_2/95% (v/v) O_2 whilst hypercapnia was induced by pre-exposing cells to 10% (v/v) CO_2/90% (v/v) O_2 for 20 mins prior to being studied. Apical [Cl^-] was reduced to 40mM and cells were stimulated with either adenosine (10μM) or IBMX (1mM) before addition of apical $CFTR_{inh}$-172 (20μM) and basolateral bumetanide (25μM). (A) displays the maximal adenosine-stimulated increase in I_{sc} and (B) displays 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) displays the maximal IBMX-stimulated increase in I_{sc} and (D) displays the rate of increase in IBMX-stimulated I_{sc}. Data represents mean ± S.E.M.; *n*=3 for normocapnia and *n*=4 for hypercapnia.

Figure 4. *The effect of hypercapnia on cAMP-dependent transepithelial anion secretion is independent of CO_2-induced intracellular acidosis*. (A) shows a representative experiment in which Calu-3 cells were gassed with 5% (v/v) CO_2/95% (v/v) O_2 and exposed to 40mM sodium acetate and pHi was measured using fluorescent microscopy. (B) summarizes the magnitude of the intracellular acidosis resulting from either 10% CO_2 or sodium acetate. Data represents mean ± S.E.M., *n*=60 for 10% CO_2; *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 $CFTR_{inh}$-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% CO_2 and 10% CO_2 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% CO_2 and 10% CO_2 and the relative expression of biotinlayed 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 $CFTR_{inh}$-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 inhibitied by $CFTR_{inh}$-172 (F) are displayed. * = significant effect of hypercapnia ($p<0.05$). Data represents mean ± S.E.M.; *n* = 6 for each.

Figure 1

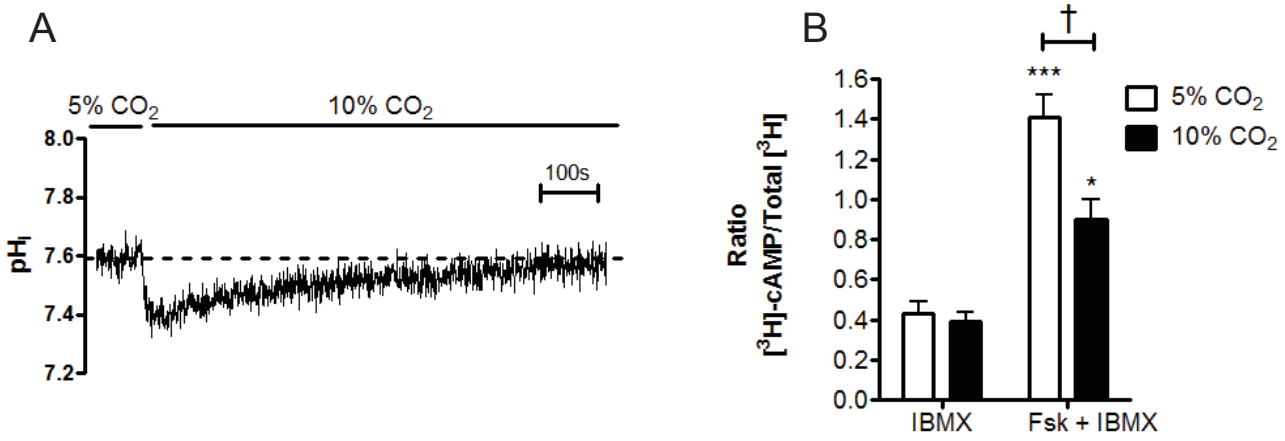

A
5% CO2
10% CO2
100s
pHi
8.0
7.8
7.6
7.4
7.2
B
†
***
*
Ratio
[3H]-cAMP/Total [3H]
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5% CO2
10% CO2
IBMX
Fsk + IBMX

Figure 2

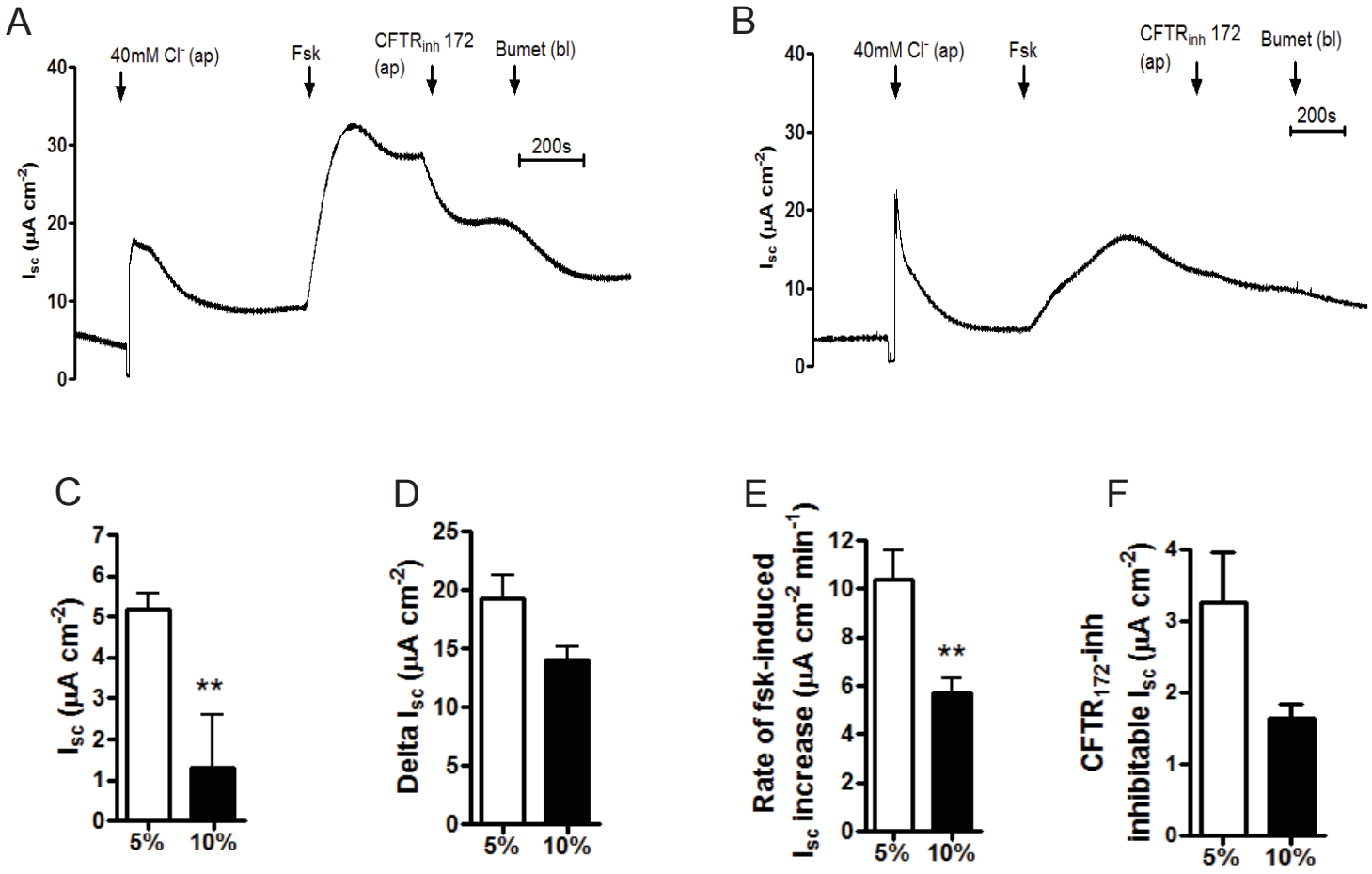

A
40mM Cl- (ap)
Fsk
CFTRinh 172 (ap)
Bumet (bl)
200s
Isc (µA cm-2)
B
40mM Cl- (ap)
Fsk
CFTRinh 172 (ap)
Bumet (bl)
200s
Isc (µA cm-2)
C
Isc (µA cm-2)
**
5%
10%
D
Delta Isc (µA cm-2)
5%
10%
E
Rate of fsk-induced Isc increase (µA cm-2 min-1)
**
5%
10%
F
CFTR172-inh inhibitable Isc (µA cm-2)
5%
10%

Figure 3

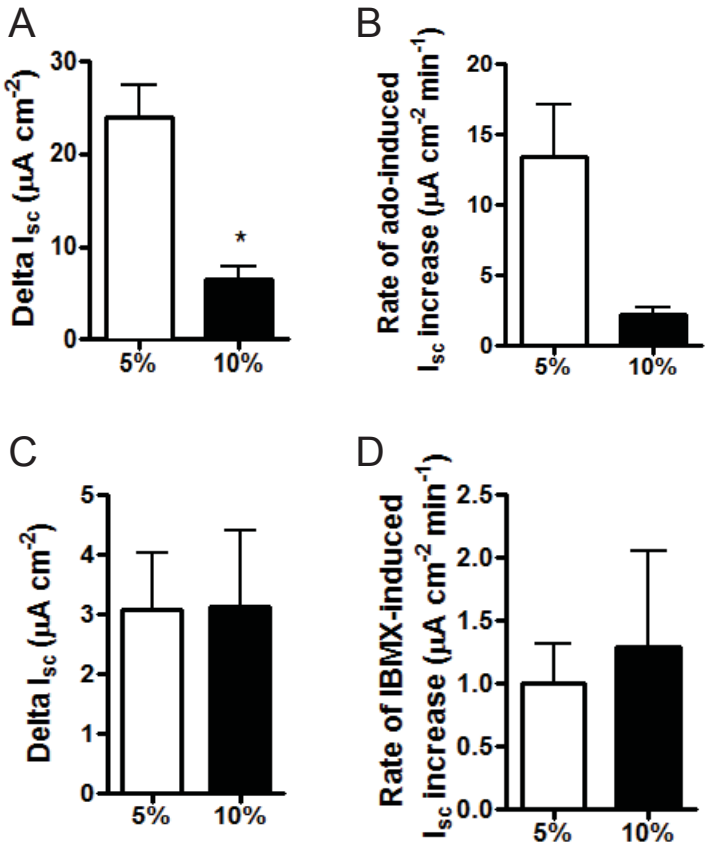

A
Delta I_{sc} (μA cm^{-2})
30
20
10
0
*
5%
10%
B
Rate of ado-induced I_{sc} increase (μA cm^{-2} min^{-1})
20
15
10
5
0
5%
10%
C
Delta I_{sc} (μA cm^{-2})
5
4
3
2
1
0
5%
10%
D
Rate of IBMX-induced I_{sc} increase (μA cm^{-2} min^{-1})
2.5
2.0
1.5
1.0
0.5
0.0
5%
10%

Figure 4

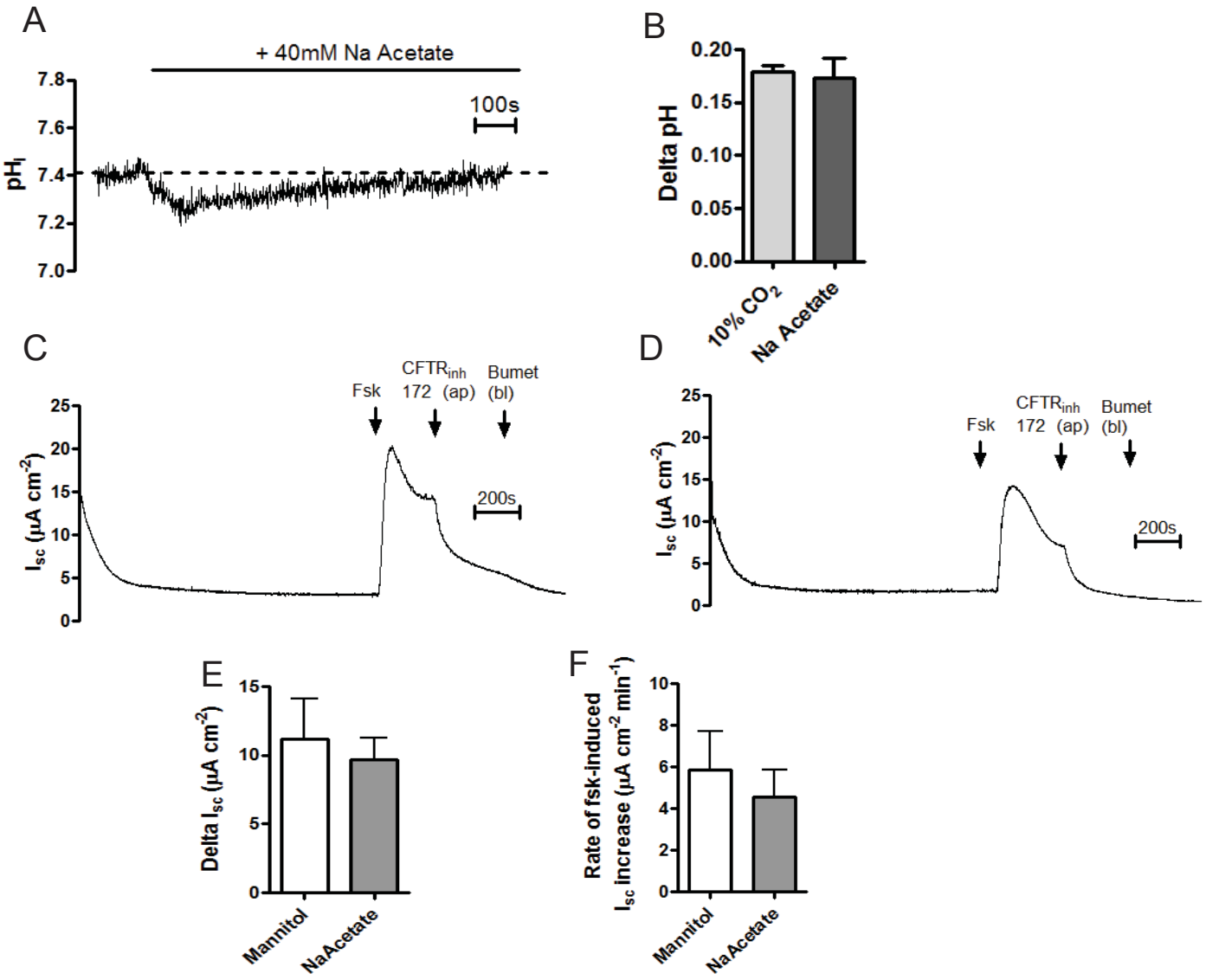

A
+ 40mM Na Acetate
100s
pH_i
7.8
7.6
7.4
7.2
7.0
B
Delta pH
0.20
0.15
0.10
0.05
0.00
10% CO2
Na Acetate
C
Fsk
CFTRinh 172 (ap)
Bumet (bl)
200s
Isc (µA cm-2)
25
20
15
10
5
0
D
Fsk
CFTRinh 172 (ap)
Bumet (bl)
200s
Isc (µA cm-2)
E
Delta Isc (µA cm-2)
15
10
5
0
Mannitol
NaAcetate
F
Rate of fsk-induced Isc increase (µA cm-2 min-1)
10
8
6
4
2
0
Mannitol
NaAcetate

Figure 5

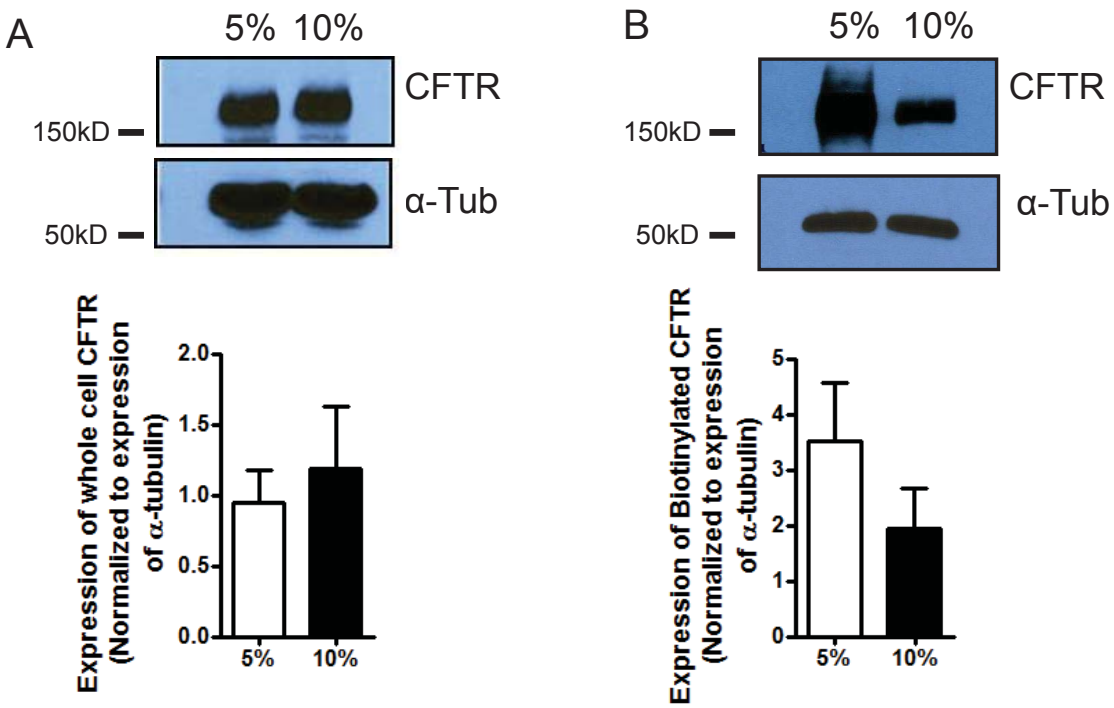

A
5% 10%
CFTR
150kD
α-Tub
50kD
Expression of whole cell CFTR (Normalized to expression of α-tubulin)
2.0
1.5
1.0
0.5
0.0
5%
10%
B
5% 10%
CFTR
150kD
α-Tub
50kD
Expression of Biotinylated CFTR (Normalized to expression of α-tubulin)
5
4
3
2
1
0
5%
10%

Figure 6

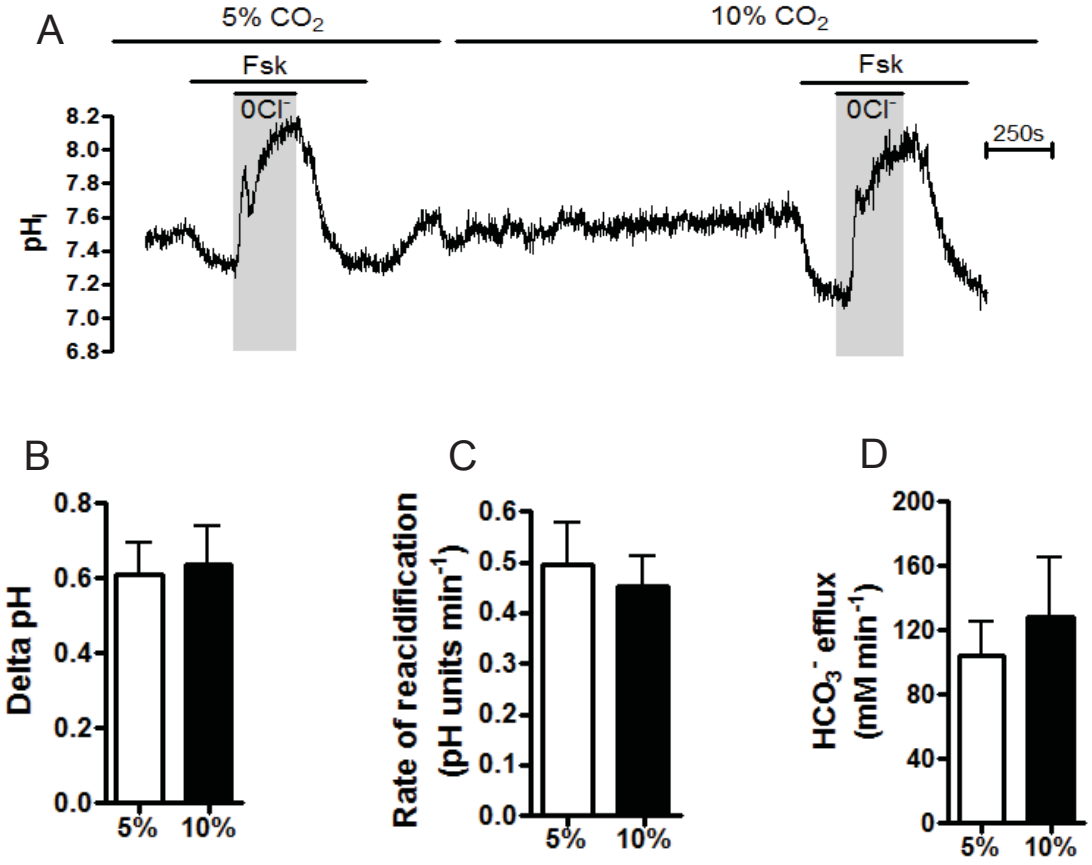

A
5% CO2
10% CO2
Fsk
0Cl-
Fsk
0Cl-
250s
pHi
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
B
Delta pH
0.8
0.6
0.4
0.2
0.0
5%
10%
C
Rate of reacidification (pH units min-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
5%
10%
D
HCO3- efflux (mM min-1)
200
160
120
80
40
0
5%
10%

Figure 7

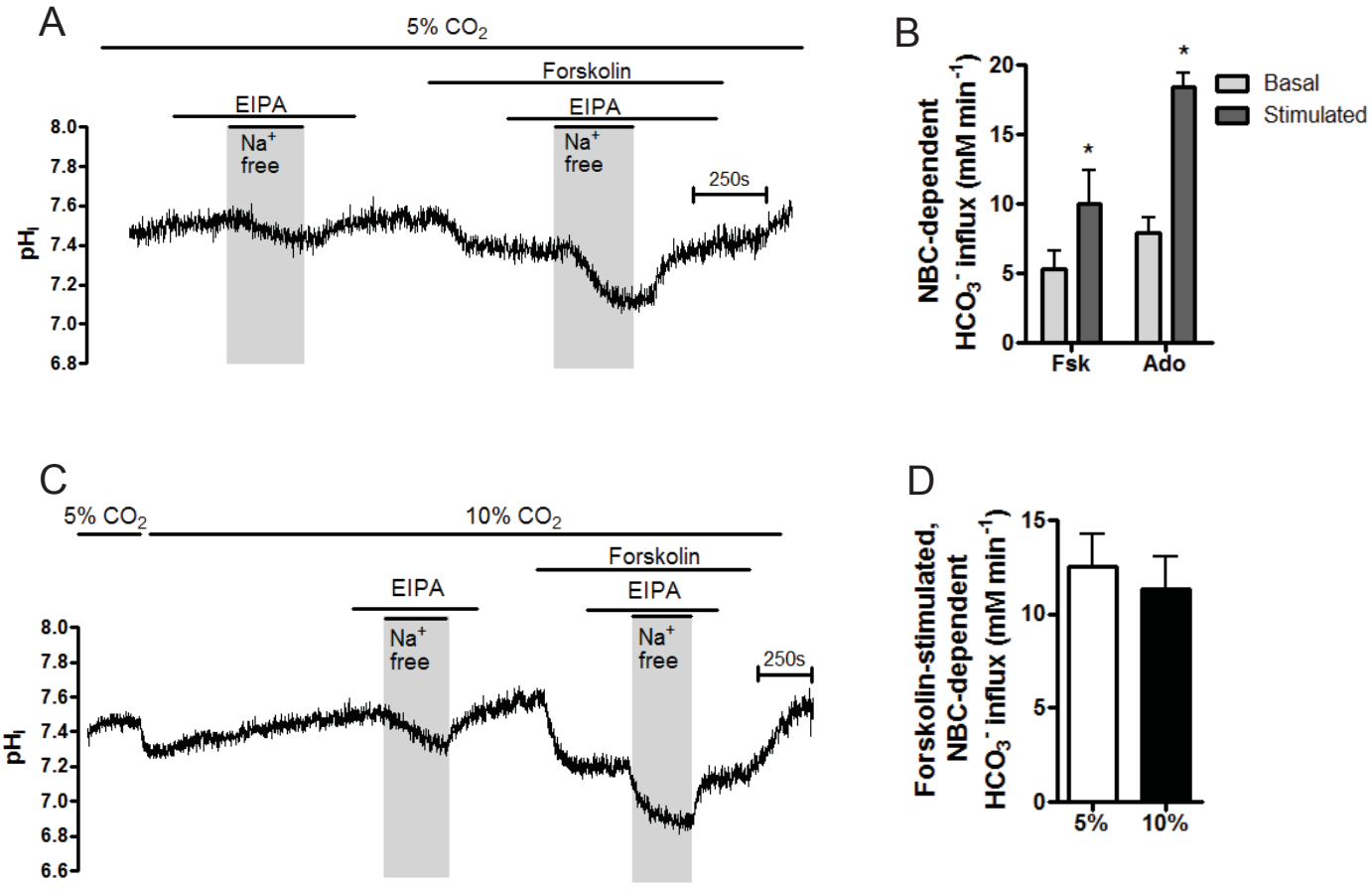

A
5% CO2
Forskolin
EIPA
EIPA
Na+ free
Na+ free
250s
pHi
8.0
7.8
7.6
7.4
7.2
7.0
6.8
B
NBC-dependent HCO3- influx (mM min-1)
20
15
10
5
0
*
*
Basal
Stimulated
Fsk
Ado
C
5% CO2
10% CO2
Forskolin
EIPA
EIPA
Na+ free
Na+ free
250s
pHi
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
D
Forskolin-stimulated, NBC-dependent HCO3- influx (mM min-1)
15
10
5
0
5%
10%

Figure 8

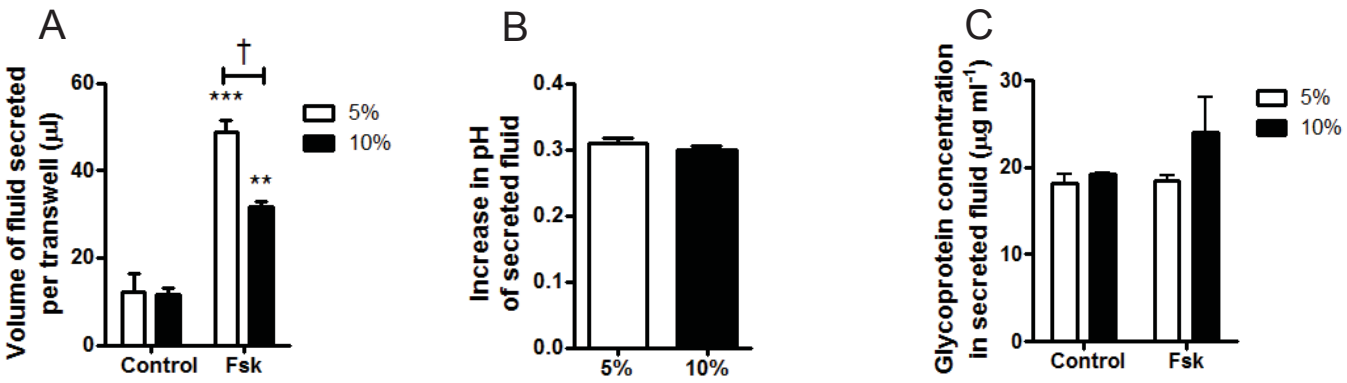
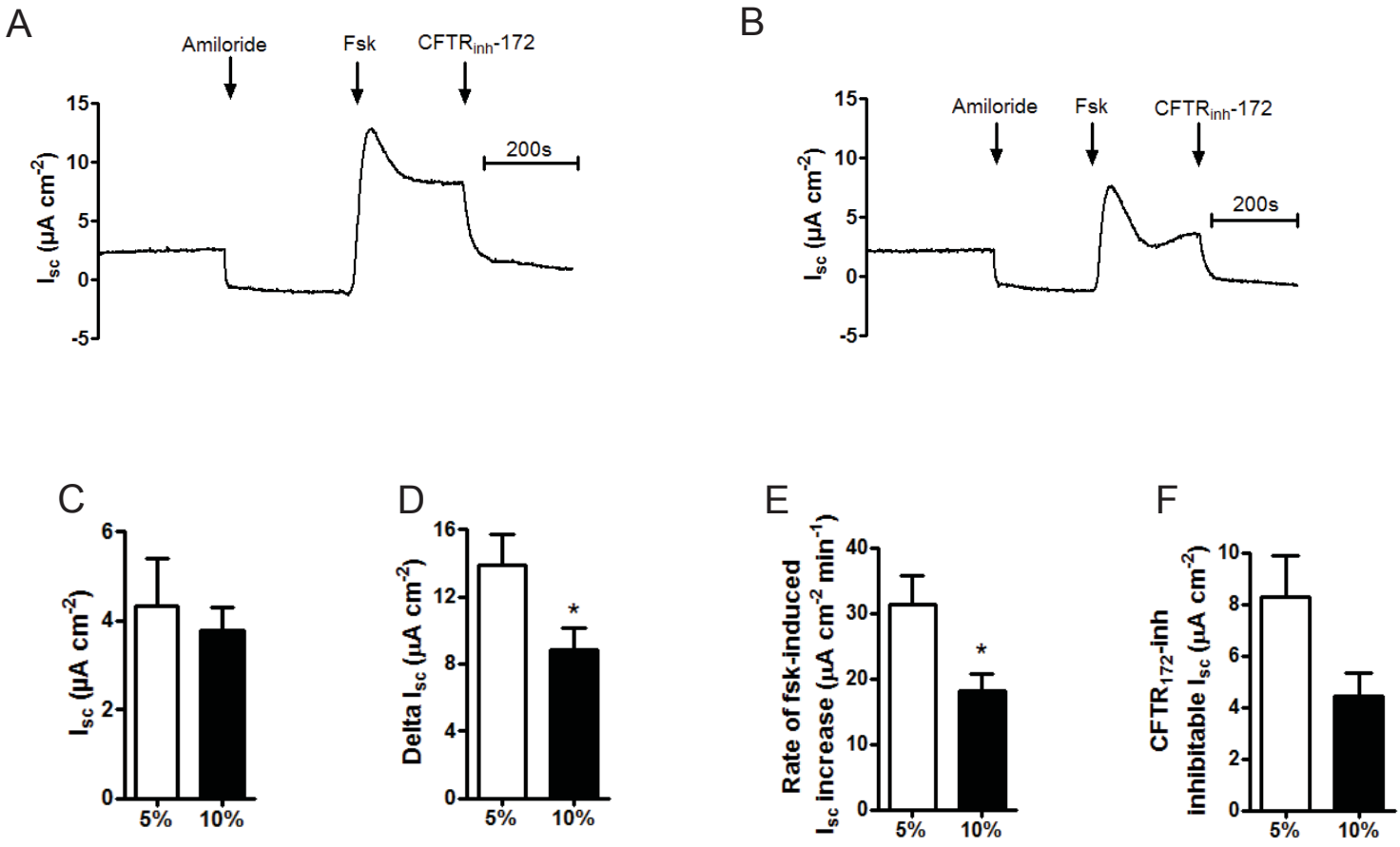

A
Volume of fluid secreted per transwell (µl)
60
40
20
0
***
**
†
Control
Fsk
5%
10%
B
Increase in pH of secreted fluid
0.4
0.3
0.2
0.1
0.0
5%
10%
C
Glycoprotein concentration in secreted fluid (µg ml-1)
30
20
10
0
Control
Fsk
5%
10%

Figure 9


A
Amiloride
Fsk
CFTRinh-172
200s
Isc (µA cm-2)
B
Amiloride
Fsk
CFTRinh-172
200s
Isc (µA cm-2)
C
Isc (µA cm-2)
5%
10%
D
Delta Isc (µA cm-2)
*
5%
10%
E
Rate of fsk-induced Isc increase (µA cm-2 min-1)
*
5%
10%
F
CFTR172-inh inhibitable Isc (µA cm-2)
5%
10%