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RESEARCH PAPER

Hydrogen peroxide stimulation of CFTR reveals an Epac-mediated, soluble AC-dependent cAMP amplification pathway common to GPCR signalling

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BACKGROUND AND PURPOSE

H_2O_2 is widely understood to regulate intracellular signalling. In airway epithelia, H_2O_2 stimulates anion secretion primarily by activating an autocrine PGE_2 signalling pathway via EP_4 and EP_1 receptors to initiate cytic fibrosis transmembrane regulator (CFTR)-mediated Cl^- secretion. This study investigated signalling downstream of the receptors activated by H_2O_2.

EXPERIMENTAL APPROACH

Anion secretion by differentiated bronchial epithelial cells was measured in Ussing chambers during stimulation with H_2O_2, an EP_4 receptor agonist or β_2-adrenoceptor agonist in the presence and absence of inhibitors of ACs and downstream effectors. Intracellular calcium ($[Ca^{2+}]_I$) changes were followed by microscopy using fura–2-loaded cells and PKA activation followed by FRET microscopy.

KEY RESULTS

Transmembrane adenylyl cyclase (tmAC) and soluble AC (sAC) were both necessary for H_2O_2 and EP_4 receptor-mediated CFTR activation in bronchial epithelia. H_2O_2 and EP_4 receptor agonist stimulated tmAC to increase exchange protein activated by cAMP (Epac) activity that drives PLC activation to raise $[Ca^{2+}]_i$ via Ca^{2+} store release (and not entry). Increased $[Ca^{2+}]_i$ led to sAC activation and further increases in CFTR activity. Stimulation of sAC did not depend on changes in $[HCO_3^-]$. Ca^{2+}-activated apical $K_{Ca}1.1$ channels and cAMP-activated basolateral $K_V7.1$ channels contributed to H_2O_2-stimulated anion currents. A similar Epac-mediated pathway was seen following β_2-adrenoceptor or forskolin stimulation.

CONCLUSIONS AND IMPLICATIONS

H_2O_2 initiated a complex signalling cascade that used direct stimulation of tmACs by Gαs followed by Epac-mediated Ca^{2+} crosstalk to activate sAC. The Epac-mediated Ca^{2+} signal constituted a positive feedback loop that amplified CFTR anion secretion following stimulation of tmAC by a variety of stimuli.

Abbreviations

AC, adenylyl cyclase; 2-APB, 2-aminoethyl diphenyl borinate; 8-pCPT-2′-O-Me-cAMP, 8-pCPT-2′-O-methyl- adenosine 3′,5′-cyclic monphosphate-acetoxy methyl ester; ALI, air–liquid interface; ESI-09, 3-[5-(tert.-butyl)isoxazol-3-yl]-2-[2-(3-chlorophenyl)hydrazono]-3-oxopropanenitrile; I_{SC}, short circuit current; KH, Krebs–Henselait buffer; NHBE, normal human bronchial epithelia

Tables of Links

TARGETS	
GPCRs[a]	**Transporters**[d]
β_2-adrenoceptor	Ca^{2+}-ATPases
EP_1 receptor	**Enzymes**[e]
EP_4 receptor	Adenylyl cyclases
Ligand-gated ion channels[b]	COX
IP_3 receptor	Epac
Ryanodine receptor	PKA
Ion channels[c]	PLCε
CFTR	
$K_{Ca}1.1$	
$K_V7.1$	

LIGANDS	
2-APB	Clotrimazole
8-pCPT-2′-O-Me cAMP	Forskolin
Albuterol	H_2O_2
Amiloride	IP_3
ATP	Paxilline
cAMP	PGE_2
Clofilium	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d,e]Alexander *et al.*, 2013a,b,c,d,e).

Introduction

Ion secretion and absorption by bronchial epithelia are essential elements of the mucociliary clearance mechanism, providing the osmotic conditions needed for appropriate water movement across the mucosa that results in ample luminal fluid for ciliary beating and mucus hydration (Boucher, 2007). Control of ion fluxes at the bronchial mucosal surface is complex and multifaceted. H_2O_2 is known to either directly or indirectly regulate several important components at the apical side of bronchial epithelial cells including a cAMP-regulated epithelial cell membrane Cl^- channel [cytic fibrosis transmembrane regulator (CFTR)] (Cowley and Linsdell, 2002b), and Na^+ and K^+ channels (Liu *et al.*, 2009; 2010; Ma, 2011; Downs *et al.*, 2013). In airway epithelia, H_2O_2 stimulates anion secretion primarily by activating an autocrine PGE_2 signalling pathway via EP_4 and EP_1 receptors, leading to CFTR-mediated Cl^- secretion (Conner *et al.*, 2010; 2013; Jones *et al.*, 2012). Pharmacological analysis of H_2O_2 stimulation shows that the majority of the anion secretion response occurs through CFTR and that EP_4 receptor signalling is the predominant stimulator as the majority of the H_2O_2-induced response was blocked by EP_4 receptor antagonist (Conner *et al.*, 2013). Both EP_1 and EP_4 receptors are GPCRs, with EP_4 believed to act primarily through Gαs and EP1 to act primarily through Gq. As CFTR anion secretion is regulated by PKA phosphorylation, it is expected that H_2O_2 stimulation of CFTR will mostly occur through Gαs activation of transmembrane AC (tmAC), but that Gq-mediated increased intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_I$) will also activate, although to a lesser extent, the Ca^{2+} activated tmACs and soluble AC (sAC) (Litvin *et al.*, 2003).

A complex set of regulatory signals control CFTR gating. PKA phosphorylation is a key element, although other kinases and phosphatases (e.g. Chappe *et al.*, 2003; Billet *et al.*, 2013) are essential. CFTR is regulated by interaction with a large number of other proteins found in complexes containing CFTR (e.g. Kunzelmann and Mehta, 2013). Regulation of CFTR by extracellular signals is further adjusted via compartmentalization of cAMP signalling by actin cytoskeleton (Fanelli *et al.*, 2008) and through co-localization with A-kinase anchor proteins and associated PDE activity (e.g. Sun *et al.*, 2000).

The studies of H_2O_2 and GPCR-mediated activation of CFTR, described here, show that H_2O_2-mediated signalling to alter anion secretion occurs through both tmAC and sAC activities. In addition, the data show that cAMP not only drives PKA activity directly, but also leads to increased $[Ca^{2+}]_i$ via exchange protein activated by cAMP (Epac) that, in turn, provides an amplified cAMP response through further increases in Ca^{2+}-stimulated ACs, including sAC.

Methods

Solutions

For intact airway epithelial cells, the apical and basolateral bath solution (Krebs–Henseleit, KH) consisted of: 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $NaH_2PO_4{\cdot}H_2O$, 1.2 mM $CaCl_2{\cdot}2\ H_2O$, 5.5 mM glucose, pH 7.35 when gassed with 95% O_2–5% CO_2. For permeabilization of the basolateral membrane, the apical solution consisted of: 145 mM NaGluconate, 3.3 mM NaH_2PO_4, 0.8 mM Na_2HPO4, 1.2 mM $MgCl_2{\cdot}6\ H_2O$, 4 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.35. The corresponding basolateral solution consisted of: 145 mM NaCl, 3.3 mM NaH_2PO_4, 0.8 mM Na_2HPO_4, 1.2 mM $MgCl_2{\cdot}6\ H_2O$, 1.2 mM $CaCl_2$, 10 mM mannitol and 10 mM HEPES, pH 7.35 (Cowley and Linsdell, 2002b). Bicarbonate-free buffer consisted of: 133 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $NaH_2PO_4{\cdot}H_2O$, 1.2 mM $CaCl_2{\cdot}2\ H_2O$, 5.5 mM glucose and 10 mM HEPES, pH 7.35.

Cell culture and lentivirus infection

All experiments used fully differentiated normal human bronchial epithelial (NHBE) cells in air–liquid interface (ALI)

culture. Human lungs, not to be used for transplant, were obtained from the Life Alliance Organ Recovery Association following appropriate consent as determined by the Institution Review Board and in accordance with the Declaration of Helsinki. Cells were isolated from human lungs, cultured and differentiated at an ALI as described previously (Nlend *et al.*, 2002; Fulcher *et al.*, 2005). All experiments were performed using date- and lung-matched cultures. Cells were judged to be differentiated by the presence of beating cilia and mucus secretion (14–28 days at the ALI). For expression of shRNA, undifferentiated cells were infected with pLKO.1-based third-generation lentiviruses encoding shRNA for sAC (SHC002, non-targeted; TRCN0000078370, exon 2; TRCN0000078369, exon 15; Sigma-Aldrich) and selected with 1 $\mu g \cdot mL^{-1}$ puromycin until uninfected control cultures were dead and then puromycin removed. Cultures were then redifferentiated at the ALI before analysis.

Ussing chambers

Differentiated NHBE ALI cultures on Snapwells (Corning, Inc., Corning, NY, USA) were rinsed and mounted in KH buffer in EasyMount Ussing chambers (Physiologic Instruments, San Diego, CA, USA) at 37°C. The KH buffer was maintained at pH 7.35 when gassed with 95% O_2/5% CO_2. All experiments were performed in the presence of 10 μM amiloride in the apical chamber to block effects of the sodium channel. To monitor short circuit current (I_{SC}), the transepithelial membrane potential was clamped at 0 mV with a six-channel voltage clamp (model VCC MC2, Physiologic Instruments) using Ag/AgCl electrodes in agar bridges. Signals were digitized and recorded with DAQplot software (VVI Software, College Station, PA, USA) via a LabJack A/D converter (LabJack Corp., Lakewood, CO, USA). The input resistance of each filter was measured by application of 1 mV bipolar pulses of 2 s duration. For permeabilization experiments, the appropriate solutions were bubbled with air and the cells were incubated with basolateral 100 μM nystatin before exposure to H_2O_2. Inhibitors were added 20–50 min before stimulation with H_2O_2 or agonists. For BAPTA experiments, 10 μM BAPTA-AM was added to mucosal and serosal compartments in KH buffer for 1 h before stimulation.

Microscopy

To assess changes in $[Ca^{2+}]_i$, differentiated NHBE ALI cultures were loaded with fura-2 AM (10 μM) in Dulbecco's PBS containing 1% glucose and 10% FBS for 2 h at room temperature. Cultures were washed and mounted in a perfusion chamber in KH buffer at room temperature and ratiometric images captured and quantified as described previously (Lieb *et al.*, 2002).

To assess changes in PKA activation, NHBE cells were transduced with lentiviruses expressing PKA subunit FRET sensors (Zaccolo and Pozzan, 2002) as described previously (Schmid *et al.*, 2006). Undifferentiated cultures were transduced and then redifferentiated at the ALI before experiments as fully differentiated cultures were resistant to viral transduction. Fully differentiated cultures were mounted in a perfusion chamber at room temperature and FRET signals (cyan fluorescent protein/yellow fluorescent protein ratios) were acquired and quantified using MetaFluor software as described previously (Schmid *et al.*, 2010).

Data analysis

Changes in I_{SC} were normalized to lung- and date-matched control cultures that were assayed simultaneously with experimental cultures and expressed as fraction of control ΔI_{SC}. This normalization controlled for variations in the magnitude of responses among lung donors. Replicate cultures from each lung donor and then all donors were averaged to give mean values for the fraction of control ΔI_{SC}. Mean values were compared by one-way ANOVA and if significant differences were obtained, by the Tukey's Kramer honestly significant difference test. EC_{50} and IC_{50} values were calculated by nonlinear regression fit of the log of agonist concentrations versus normalized ΔI_{SC} responses.

Reagents

All reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. DMEM, Ham's nutrient F-12 and HBSS were purchased from Gibco, Life Technologies (Grand Island, NY, USA). Clotrimazole, $CFTR_{inh}$-172, forskolin, KH7, SQ22536, MDL-12,330A (Enzo Life Sciences, Farmingdale, NY, USA), BAPTA-AM, fura-2 AM (Invitrogen, Carlsbad, CA, USA), U-73343, gallein, 3-[5-(tert.-butyl)isoxazol-3-yl]-2-[2-(3-chlorophenyl)hydrazono]-3-oxopropanenitrile (ESI-09) and 8-pCPT-2′-O-methyl- adenosine 3′,5′-cyclic monphosphate-acetoxy methyl ester (8-pCPT-2′-O-Me-cAMP) were dissolved in DMSO. Both ESI-09 and 8-pCPT-2′-O-Me-cAMP were from Biolog, (Bremen, Germany). Amiloride, H89, clofilium, 4,4′-dinitrostilbene-2,2′-disulphonic acid and $BaCl_2$ were dissolved in distilled H_2O. Cay10598 (Cayman Chemicals, Ann Arbor, MI, USA) and U-73122 were dissolved in ethanol. Paxilline and 2-aminoethyl diphenyl borinate (2-APB) were dissolved in methanol. Nystatin was dissolved in distilled H_2O (or bath solution) and sonicated before use.

Results

Role of tmACs and sAC in H_2O_2-mediated CFTR activation

H_2O_2-mediated anion secretion by bronchial epithelial cells is known to occur through stimulation of both EP_4 and EP_1 PG receptors (Joy and Cowley, 2008; Jones *et al.*, 2012; Conner *et al.*, 2013). The magnitude and duration of the H_2O_2 response depends on the concentration, with higher H_2O_2 leading to transient changes and lower H_2O_2 leading to sustained increases in I_{SC} (Conner *et al.*, 2013), and both sustained and transient changes occurring through activation of COX and stimulation of EP_1 and EP_4 receptors to increase CFTR activity (Joy and Cowley, 2008; Jones *et al.*, 2012; Conner *et al.*, 2013). Using receptor antagonists, EP_4 stimulation was shown to contribute the larger proportion of CFTR activation (Conner *et al.*, 2013). To examine the EP_4 receptor signalling mechanisms that lead to H_2O_2-mediated CFTR activation, MDL-12,330A and KH7, inhibitors of tmAC and sAC, respectively (Hess *et al.*, 2005; Bitterman *et al.*, 2013), were used to block H_2O_2-stimulated CFTR currents (in the presence of amiloride to eliminate Na^+ channel contributions) in fully differentiated NHBE cells mounted in Ussing chambers (Figure 1). MDL-12,330A inhibited H_2O_2-mediated stimulation of CFTR currents with an IC_{50} of 6.2 μM that was nearly

identical to its inhibition of forskolin stimulation (IC_{50} = 7.3 μM) suggesting that inhibition of H_2O_2 stimulation occurred by blocking tmACs (Figure 1A). However, KH7 also inhibited 1 mM H_2O_2 stimulation of anion secretion with an IC_{50} = 2 μM, similar to the published values for sAC (Hess *et al.*, 2005; Bitterman *et al.*, 2013). Similar results were obtained using a lower concentration of H_2O_2 (Supporting Information Fig. S1). KH7 did inhibit forskolin stimulation of CFTR currents only at higher concentrations and did not block all activity (IC_{50} = 26 μM; Figure 1B). To rule out the possibility that MDL-12,330A also inhibits sAC with an IC_{50} equivalent to its action against tmAC and that sAC is responsible for all activation of CFTR via H_2O_2, SQ 22536 was used and showed that the H_2O_2 response was also sensitive to this tmAC inhibitor (data not shown). These data suggest that inhibition of tmACs and sAC were involved in the H_2O_2 response and that inhibition of either could block the majority of the response.

To confirm a role for sAC, undifferentiated NHBE cells were infected with lentivirus encoding sAC-specific shRNAs, directed to either exon 2 or exon 15, or non-targeted shRNA. Following redifferentiation, the CFTR response to H_2O_2 of NHBE cultures infected with shRNA targeted to sAC exon 15 was reduced when compared with control cultures infected with non-targeted shRNA virus. Exon 2 sAC shRNA was not different from the control (Figure 1C; $P < 0.05$, $n = 5$ cultures from each of two lung donors). sAC mRNA undergoes a variety of alternative splices (Chen *et al.*, 2014) and is a rare message in bronchial epithelia that renders assessment of both mRNA and protein reductions after knock-down impossible. It is not clear which alternatively spliced form of sAC plays a role in the response, and the lack of an effect by the exon 2-targeted shRNA may simply be due to the efficacy of this shRNA sequence. However, the clear reduction of the physiological response to H_2O_2 following infection with shRNA to sAC strongly supports the pharmacological data mentioned earlier that indicated sAC plays an essential role in CFTR stimulation in addition to tmAC.

H_2O_2 stimulates sAC through increased $[Ca^{2+}]_i$

sAC activity is stimulated by both Ca^{2+} and HCO_3^- (Litvin *et al.*, 2003). H_2O_2 leads to increases in $[Ca^{2+}]_i$ in a number of cell types (e.g. Hayashi *et al.*, 1989; Rice *et al.*, 1992; Meyer *et al.*, 1996; Nakazaki *et al.*, 2000). To assess whether H_2O_2 treatment of NHBE cells raised $[Ca^{2+}]_i$, fluorescence changes in fura–2-loaded cells were followed by microscopy and showed a transient increase in $[Ca^{2+}]_i$ (Figure 2A). As sAC is stimulated by increases in $[Ca^{2+}]_i$, NHBE cultures were loaded with BAPTA-AM before being placed into Ussing chambers and treated with H_2O_2. BAPTA loading significantly reduced H_2O_2-mediated CFTR activation (Figure 2B and C), supporting the idea that increased $[Ca^{2+}]_i$ was the signal for sAC activation. The PLC inhibitor U-73122, but not the mostly inactive analogue U-73343 (e.g. Horowitz *et al.*, 2005), blocked H_2O_2-mediated CFTR activity increases at the expected concentration (Figure 2D and E). The IP_3 receptor antagonist 2-APB also blocked H_2O_2 stimulation (Figure 2F) suggesting that IP_3-mediated Ca^{2+} release was an important element in sAC activation following H_2O_2. Interestingly, neither nominal outside Ca^{2+} nor exchange of Ca^{2+} for Sr^{2+} in external buffers reduced the H_2O_2-mediated CFTR current, suggesting that Ca^{2+} entry did not play a significant role in increasing $[Ca^{2+}]_i$ (Figure 2F).

CO_2 and HCO_3^- also stimulate sAC activity (Litvin *et al.*, 2003). To evaluate their role in H_2O_2-mediated sAC activation, H_2O_2 stimulation of CFTR activity in Ussing chambers was measured in HCO_3^- free buffer in the presence and absence of acetazolamide (1 mM) to inhibit carbonic anhydrase. Neither HCO_3^--free buffers (Figure 3) nor carbonic anhydrase inhibition (data not shown) reduced the amplitude of H_2O_2-stimulated CFTR $I_{SC,}$ although the time to return to baseline was extended compared with HCO_3^--containing buffers. However, KH7 similarly reduced anion currents in these conditions compared with controls (Figure 3B). Thus, sAC stimulation after H_2O_2 treatment appeared to depend only on changes in $[Ca^{2+}]_i$ and not CO_2/HCO_3^-.

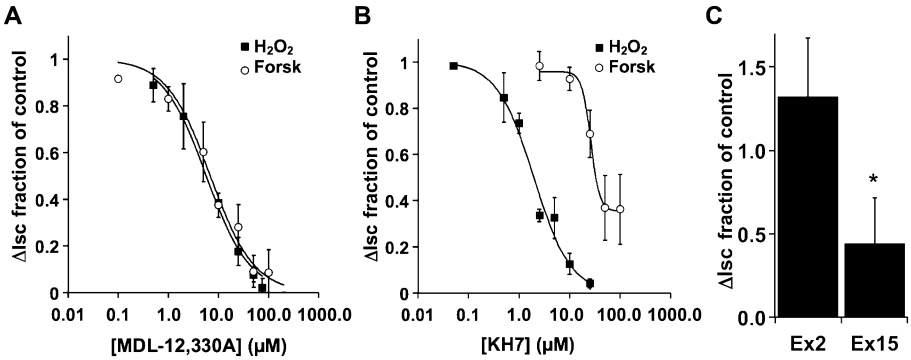

Figure 1

H_2O_2 stimulates both tmAC and sAC. Fully differentiated NHBE cells in ALI culture were mounted in Ussing chambers and stimulated with either H_2O_2 (1 mM) or forskolin (10 μM) in the presence of various concentrations of the tmAC inhibitor MDL-12,330A (A, n = 3–6 lung donors at each concentration) or various concentrations of the sAC inhibitor KH7 (B, 3–6 lung donors at each concentration). (C) NHBE cells were infected with shRNA expressing lentiviruses targeted to either exon 2 or exon 15 of sAC or with non-targeted lentiviruses. After differentiation, cultures were mounted in Ussing chambers and stimulated with H_2O_2 (1 mM). Compared with non-target controls and exon 2-targeted cultures, the response of exon 15 targeted cultures was significantly reduced (n = 5 cultures from two lung donors, *$P < 0.05$).

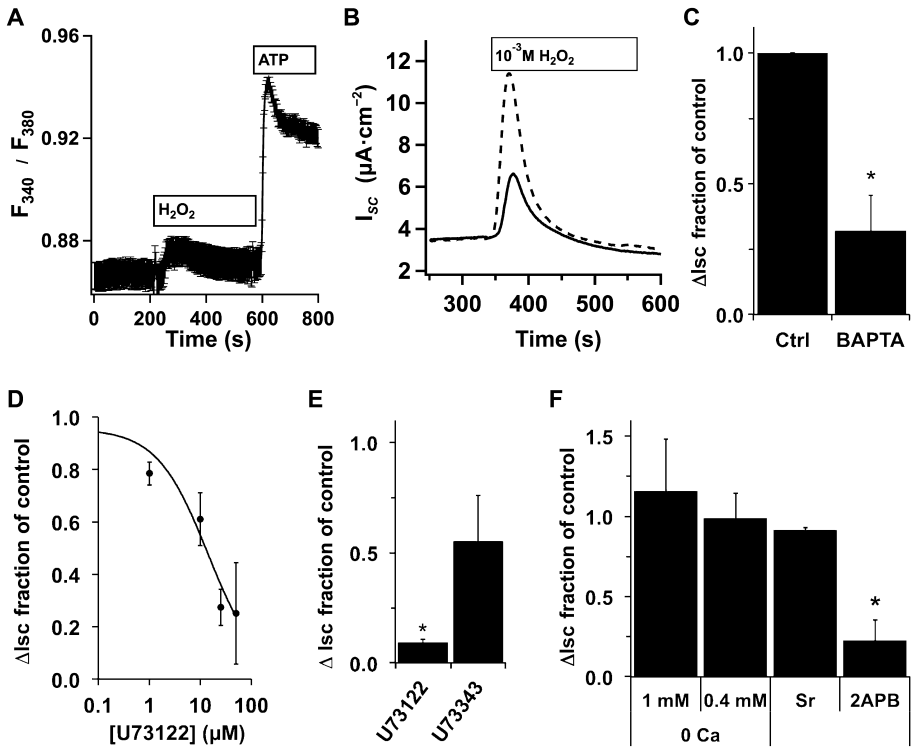

Figure 2

H_2O_2-mediated $[Ca^{2+}]_i$ increases stimulate anion secretion. (A) Fully differentiated NHBE cells in ALI culture were loaded with fura-2AM and mounted in a perfusion chamber on a Nikon E600fn microscope (Nikon Inc., Melville, NY, USA). H_2O_2 (400 μM) led to an increase in $[Ca^{2+}]_i$. Subsequent perfusion with ATP (10 μM) led to the expected robust response (ratio plotted is the mean of 16 cells from 1 donor ± SEM, representative of experiments with three individual lungs). For comparison of ATP-stimulated I_{SC} changes, see Supporting Information Fig. S3. (B) Representative I_{SC} traces of fully differentiated NHBE cells in ALI culture loaded with 25 μM BAPTA-AM (solid trace) before they were mounted in Ussing chambers and stimulated with H_2O_2 (1 mM) compared with control (unloaded cells; dashed trace). (C) BAPTA-loaded cultures had a reduced anion secretion response to 1 mM H_2O_2 (mean ± SEM, $n = 6$ lung donors, two to three cultures per donor, $^*P < 0.05$). (D) NHBE ALI cultures in Ussing chambers were pretreated with different concentrations of the PLC inhibitor U73122 and then stimulated with H_2O_2 (1 mM) in the presence of inhibitor. U73122 led to a concentration-dependent decrease in anion secretion with an apparent $IC_{50} = 10$ μM ($n = 3$–4 lung donors at each concentration). (E) Comparison of U73122 (25 μM) with the less active isomer U73343 (25 μM) showed specificity. (F) NHBE ALI cultures were mounted in Ussing chambers and stimulated with H_2O_2 (1 or 0.4 mM) in the presence or absence of extracellular Ca^{2+}, in the presence of Sr^{2+} instead of Ca^{2+} or in the presence of 2-APB (200 μM). Neither removal of Ca^{2+} nor substitution of Ca^{2+} with Sr^{2+} significantly reduced anion secretion ($n = 3$ lung donors), while addition of the IP_3 receptor antagonist 2-APB significantly reduced anion secretion ($n = 5$ lung donors, $^*P < 0.05$).

Mechanism of H_2O_2-stimulated increases in $[Ca^{2+}]_I$

The majority of the H_2O_2 stimulation of anion currents appears to be due to signalling through EP_4 receptors (Conner *et al.*, 2013) that acts primarily through Gαs to stimulate tmAC, but not sAC (Braun *et al.*, 1977; Buck *et al.*, 1999). Although the EP_1 receptor also contributes to H_2O_2 activation of CFTR via Gq stimulation of PLC, it appears to be responsible for only a small fraction of the H_2O_2 response (Conner *et al.*, 2013). Thus, it was initially surprising that KH7 inhibition of sAC blocked such a large portion of the H_2O_2-mediated stimulation of anion secretion, as sAC is insensitive to G-protein stimulation (Buck *et al.*, 1999). Several possible mechanisms were examined that might explain the apparent H_2O_2-mediated EP_4 receptor signalling through increased $[Ca^{2+}]_i$ and sAC.

PGs have been shown to signal through receptor coupling to Gβγ to regulate Ca^{2+} channels. Gallein (200 μM), an inhibitor of Gβγ activity, was added before H_2O_2 stimulation and measurement of anion secretion showed no effect with short or long pre-incubation (1–18 h; data not shown). To rule out direct H_2O_2 action on sAC or on IP_3 receptors (Zheng and Shen, 2005), cultures were treated with an EP_4 receptor agonist (Cay10598, 50 nM) instead of H_2O_2. The EP_4 receptor stimulates anion secretion primarily through CFTR (Figure 4A). KH7 or MDL-12,330A (both at 25 μM) blocked nearly 75% of the EP_4 receptor-mediated response (Figure 4B and C). The concentration-dependence of KH7 inhibition confirmed its specificity in EP_4 agonist-stimulated cells (IC_{50} = 8 μM, Figure 4D). Thus, direct H_2O_2 stimulation of downstream effectors was not responsible for KH7 sensitivity.

To demonstrate that the EP_4 receptor signalled to increase $[Ca^{2+}]_i$, NHBE cultures were loaded with fura-2 and stimulated with Cay10598. An increase in the 340 nm/380 nm fluorescence ratio confirmed that EP_4 receptor activation led to an increase in $[Ca^{2+}]_i$ (Figure 4E). In agreement, U-73122 also blocked Cay10598 responses (IC_{50} = 8 μM, data not shown). Based on the data presented earlier, a large fraction of

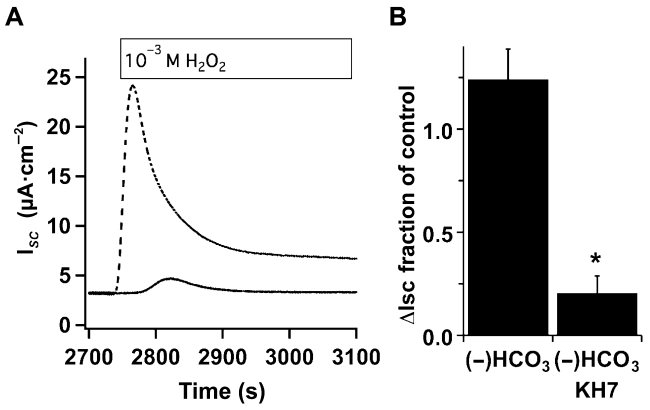

Figure 3

H_2O_2 stimulation of sAC is not HCO_3^- dependent. (A) Representative I_{SC} traces of fully differentiated NHBE cells in an ALI culture mounted in an Ussing chamber containing KH without HCO_3^-, buffered with 10 mM HEPES pH 7.4 and stimulated with 1 mM H_2O_2 in the absence (dashed trace) or presence of KH7 (solid trace, 25 μM). (B) Removal of HCO_3^- did not reduce anion secretion in response to H_2O_2 ($n = 5$ lungs), and the H_2O_2 response was sensitive to KH7 in the absence of bicarbonate ($n = 3$ lungs, $^*P < 0.05$).

H_2O_2-mediated EP_4 signalling most likely results from increased $[Ca^{2+}]_i$ that activates sAC despite EP_4 receptors coupling to Gαs and not Gq. cAMP can stimulate PLC through exchange protein directly activated by cAMP (Epac) (Schmidt *et al.*, 2001) and Epac1 is expressed in fully differentiated NHBE cells (Supporting Information Fig. S2). ESI-09, a specific Epac inhibitor (Almahariq *et al.*, 2013), blocked H_2O_2 with the expected IC_{50} (0.8 μM, Figure 4F) and also blocked EP_4 receptor stimulated anion secretion (10 μM) (Figure 4G), suggesting that increased $[Ca^{2+}]_i$ could be due to activation of PLC (shown earlier) by Epac. Pre-incubation and inclusion of ESI-09 during perfusion of fura–2-loaded cells attenuated Cay10598-mediated increases in $[Ca^{2+}]_i$ (Figure 4H and I). Thus, the data support the idea that EP_4 receptor stimulation of tmAC not only produces cAMP to stimulate CFTR anion secretion directly by PKA, but also amplifies the response by activation of Epac to increase $[Ca^{2+}]_i$ and activate sAC to further increase cAMP production. Interestingly, ESI-09 blocked a large portion of H_2O_2-stimulated anion secretion suggesting that a significant portion of PKA-stimulated CFTR activity was a result of Ca^{2+}-promoted cAMP production by sACs and tmACs.

It was possible that other Gαs-coupled receptor cAMP signals might also activate Epac and lead to Ca^{2+}-mediated sAC stimulation and similar amplification. In fact, albuterol (10 μM) stimulation of β_2-adrenoceptors to activate CFTR in NHBE cultures was partially sensitive to KH7 treatment (Figure 5A). Figure 1B shows that the KH7 partially inhibited forskolin-stimulated CFTR activity at a higher dose despite being ineffective against tmAC (Bitterman *et al.*, 2013). Given the intricacies of CFTR activity regulation by association with complexes containing regulatory components and with tmAC (Namkung *et al.*, 2010), and based on the clear specificity of KH7 for sAC over tmAC (Bitterman *et al.*, 2013), it was possible that the partial KH7 inhibition of forskolin responses reflected activation of sAC via Epac-mediated $[Ca^{2+}]_i$ transients. To assess whether forskolin-stimulated cAMP increases were due to Epac stimulation, NHBE cultures were transduced with PKA-GFP fusion proteins as FRET sensors to report PKA activation (Zaccolo and Pozzan, 2002). Increased PKA activation in response to forskolin was partially inhibited by pre-incubation with ESI-09 consistent with the hypothesis that a portion of the forskolin-induced CFTR response was Epac mediated (Figure 5B and C). Thus, the data support a role for sAC in activation of CFTR currents independent of bicarbonate changes (Wang *et al.*, 2005) and during stimulation by GPCRs rather than only contributing to basal CFTR activity (Sun and Bonanno, 2002).

To confirm a role for Epac in increasing $[Ca^{2+}]_i$ and stimulating sAC and CFTR, NHBE cultures were stimulated with the Epac agonist 8-pCPT-2′-O-Me-cAMP (20 μM) and then treated with either KH7 (Figure 5D and 5F) or CFTRinh172 (Figure 5E). Both blocked the Epac agonist-induced I_{SC}. BAPTA-AM loading of cells prior to stimulation reduced the 8-pCPT-2′-O-Me-cAMP-induced I_{SC} (Figure 5D and F). Thus, Epac1 activation appears to mimic, in part, the stimulation of I_{SC} seen following H_2O_2 treatment or stimulation of Gαs-coupled receptors.

Role of potassium channels in H_2O_2-stimulated anion secretion

Apical, PKA-stimulated CFTR Cl^- secretion from bronchial epithelia is known to rely on both apical and basolateral K^+ channel activity that provides a driving force for Cl^- secretion (e.g. Mall *et al.*, 2000; Cowley and Linsdell, 2002a,b; Manzanares *et al.*, 2011), and both increased $[Ca^{2+}]_i$ and cAMP/PKA are key in regulating these channels. Previously, others have shown that sAC stimulates basolateral K^+ secretion via $K_v7.1$ from colon epithelia following epinephrine stimulation of the β2-adrenergic receptor that also works via Gαs, but through an unknown mechanism (Halm *et al.*, 2010). Inclusion of the K^+ channel inhibitors Ba^{2+} (5 mM, nonspecific) and clofilium (100 μM, $K_v7.1$ containing complexes), but not clotrimazole (30 μM, K_{Ca} channels) in the serosal compartment of NHBE ALI cultures abrogated the H_2O_2-stimulated I_{SC} in Ussing chambers (Figure 6A and B) confirming the published data of others (e.g. Cowley and Linsdell, 2002a). However, none of the compounds added to the apical compartment had an effect on H_2O_2-stimulated currents (data not shown). Thus, as previously reported, a cAMP-activated basolateral K^+ channel (response was clofilium sensitive, Cowley and Linsdell, 2002a,b), but not a Ca^{2+}-activated K^+ channel (response was clotrimazole insensitive) was needed for apical Cl^- secretion. However, cAMP from sAC activity was not acting to stimulate basolateral K^+ channel activity following H_2O_2 treatment, as H_2O_2 stimulation of basolaterally permeabilized cells was still sensitive to KH7 (Figure 6C). Interestingly, basolateral permeabilization eliminated the MDL-12,330A sensitivity of H_2O_2-stimulated CFTR activity suggesting that H_2O_2-stimulated tmAC activity might predominantly stimulate basolateral K^+ channels (Figure 6C).

As increased $[Ca^{2+}]_i$ was seen after H_2O_2 treatment and as the $K_{Ca}1.1$ channel was recently shown to play a role in NHBE apical ion secretion (Manzanares *et al.*, 2011), the effect of a

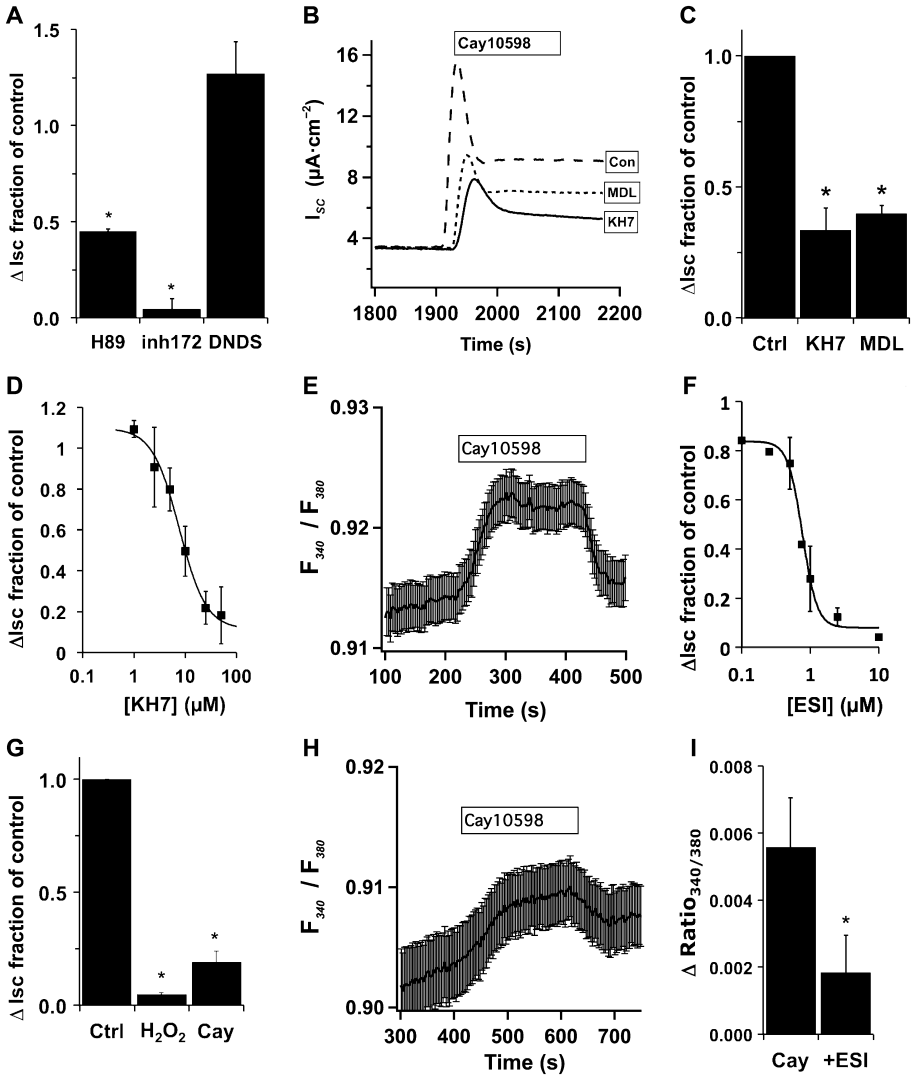

Figure 4

EP4 stimulation of CFTR activates sAC through Epac and increased $[Ca^{2+}]_i$. (A) NHBE ALI cultures in Ussing chambers were stimulated with Cay10598 (50 nM) in the presence or absence of H89 (10 μM), $CFTR_{inh}172$ (10 μM) or 4,4′-dinitro-stilbene-2,2′-disulphonic acid (DNDS; 100 μM) (n = 4 lung donors for each inhibitor, *P < 0.05). Both the kinase inhibitor H89 and the CFTR inhibitor blocked EP_4 receptor-mediated anion secretion, while DNDS has no effect, consistent with CFTR activation. (B) I_{SC} traces of NHBE ALI cultures mounted in Ussing chambers and stimulated with Cay10598 (50 nM) in the presence or absence of KH7 (25 μM) or MDL12,330A (25 μM). (C) Anion secretion was significantly reduced by each inhibitor (n = 5 lung donors, *P < 0.05). (D) NHBE ALI cultures were stimulated with Cay10598 in the presence of different concentrations of KH7 (n = 3–6 lung donors at each concentration, apparent IC_{50} = 8 μM). (E) NHBE ALI cultures were loaded with fura2-AM, mounted in a perfusion chamber and imaged by epifluorescence microscopy. Addition of Cay10598 (100 nM) to the perfusate increased $[Ca^{2+}]_i$ that returned towards baseline after removal of the agonist (representative trace is the mean fura-2 ratio recorded from regions of interest in 16 cells from a single lung donor ± SEM, and is representative of experiments with three individual donors). (F) The Epac inhibitor ESI-09 blocked H_2O_2-induced changes in I_{SC} with an apparent IC_{50} = 0.8 μM (n = 1–5 lung donors at each concentration), and ESI-09 (10 μM) attenuated Cay10598-induced anion secretion (G; n = 4 lung donors, *P < 0.05 compared with control no ESI-09). (H) Pretreatment with ESI-09 (10 μM) also attenuated changes in Cay10598-induced fura-2 fluorescence (representative trace of the mean fura-2 ratio recorded from regions of interest in 19 cells from a single donor ± SEM, and is representative of experiments with three individual donors). (I) Cay10598-induced fura-2 fluorescence changes were significantly altered by ESI-09 (10 μM; n = 4 lung donors, P < 0.05).

specific $K_{Ca}1.1$ channel blocker, paxilline, was tested [the $K_{Ca}1.1$ channel is insensitive to Ba^{2+} (Miller *et al.*, 1987; Neyton and Miller, 1988)]. Paxilline (4 μM) blocked about 30% of the H_2O_2-stimulated I_{SC} when included in the mucosal compartment (Figure 6B) suggesting that the $K_{Ca}1.1$ channel was activated and played a role in H_2O_2-stimulated apical Cl^- secretion. As expected from previous reports, paxilline only inhibited a fraction of the H_2O_2-stimulated current. These data suggest that in bronchial epithelia sAC does not play a role in basolateral K^+ secretion as shown for intestinal epithelia, and that apical $K_{Ca}1.1$ channels may be stimulated by increased $[Ca^{2+}]_i$ during H_2O_2 stimulation.

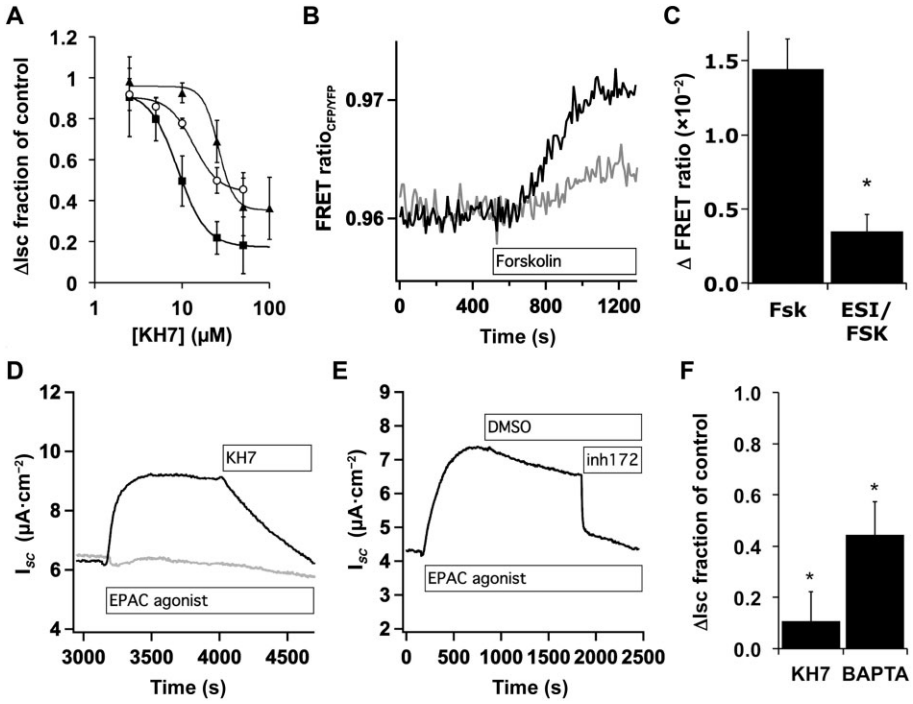

Figure 5

Epac inhibition partially blocks forskolin stimulation of CFTR. (A) NHBE ALI cultures in Ussing chambers were stimulated with Cay10598 (100 nM, squares), albuterol (10 μM, circles) or forskolin (10 μM, triangles) in the presence of KH7. Forskolin trace is from Figure 1A. Values plotted are the mean ± SEM, n = 3–6 lung donors at each point. (B) NHBE ALI cultures that were transduced with lentiviruses encoding fluorescent PKA subunits, mounted in a perfusion chamber in the presence or absence of ESI-09 (10 μM) and imaged by epifluorescence microscopy during forskolin (10 μM) stimulation. Shown are example traces from a single control cell (black trace) and a single-cell pretreated with ESI-09 (grey trace). (C) Maximum forskolin-induced changes in the FRET ratio are shown with and without ESI-09 (mean ± SEM, n = 17, eight to nine cells from each of two donors). (D, E) NHBE ALI cultures in Ussing chambers were stimulated with 8-pCPT-2′-O-Me-cAMP (20 μM) and the increased I_{SC} was blocked by addition of KH7 (25 μM, D), by BAPTA loading (D, grey trace) and by CFTRinh172 (5 μM, E). (F) NHBE ALI cultures in Ussing chambers stimulated with 8-pCPT-2′-O-Me-cAMP and treated with KH7 or were stimulated with 8-pCPT-2′-O-Me-cAMP with or without loading with BAPTA-AM (n = 3 lung donors).

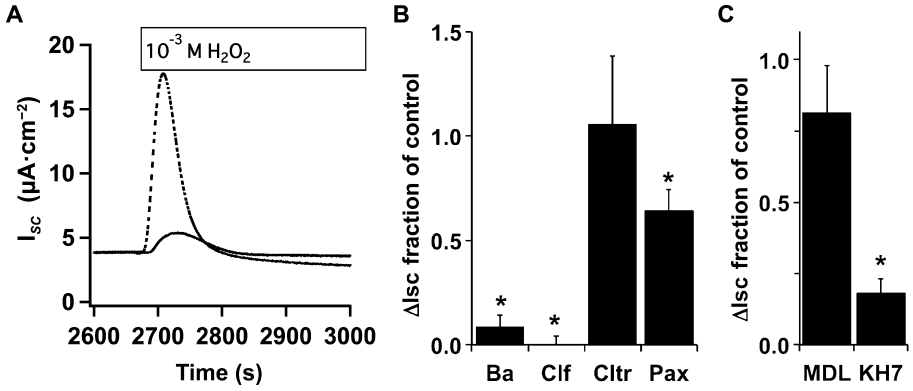

Figure 6

H_2O_2-mediated stimulation of CFTR via sAC occurs in basolaterally permeabilized cells. (A) Representative I_{SC} traces of NHBE cells in ALI culture mounted in Ussing chambers and stimulated with H_2O_2 (1 mM) with (solid trace) or without Ba^{2+} (dashed trace, 5 mM). (B) NHBE ALI cultures mounted in Ussing chambers and stimulated with H_2O_2 in the presence or absence of basolateral Ba^{2+} (5 mM), clofilium (100 μM), clotrimazole (30 μM) or apical paxilline (4 μM). Compared with controls basolateral Ba^{2+} and clofilium and apical paxilline blocked H_2O_2 responses while clotrimazole had no effect (n = 5–8 lung donors, *P < 0.05). (C) NHBE cells in ALI culture were mounted in buffer containing 100 μM nystatin to permeabilize the basolateral membrane and with or without MDL-12,330A (75 μM) or KH7 (20 μM). After stabilization of baseline I_{SC}, H_2O_2 was added to the apical compartment. KH7 inhibited H_2O_2 stimulation while MDL-12,330A inhibition was reduced by basolateral membrane permeabilization (n = 3 lung donors, *P < 0.05 compared with controls).

Discussion and conclusions

H_2O_2 stimulates bronchial epithelial cells to increase CFTR anion secretion and presumably increase available fluid on the mucosal surface. H_2O_2 is produced by Duox1 and Duox2 in NHBE cells and the H_2O_2 concentrations in the range used in these experiments are possibly encountered during inflammation and/or with impaired H_2O_2 consumption (El-Chemaly *et al.*, 2003; Conner *et al.*, 2013). H_2O_2 is produced as a substrate for lactoperoxidase (Conner *et al.*, 2002; Geiszt *et al.*, 2003) and also serves to influence signalling in cells (Strengert *et al.*, 2014). During periods of high H_2O_2 production by Duox (Harper *et al.*, 2005; Gattas *et al.*, 2009) or loss of lactoperoxidase activity (e.g. following cigarette smoke exposure Reznick *et al.*, 2003), increased H_2O_2 may therefore stimulate anion secretion as a compensatory mechanism to maintain adequate fluid levels at the airway surface.

H_2O_2 stimulation operates primarily through an autocrine pathway that activates EP_4 receptors and to a lesser extent through activation of EP_1 receptors (Conner *et al.*, 2013). The present study demonstrates that both tmAC and sAC are activated by H_2O_2 through these G-protein coupled PG receptors and that the EP_4 receptor, which primarily signals through Gαs, also activates sAC through Epac-stimulated Ca^{2+} crosstalk which serves to amplify cAMP signalling in a positive feedback loop to further increase CFTR anion secretion (Figure 7). The studies also suggest that, in bronchial epithelial cells, stimulation of β_2-adrenoceptor results in tmAC-catalysed cAMP increases to activate Epac and increase $[Ca^{2+}]_i$ to stimulate sAC and thus extends the previously reported effects of Epac after β_2-adrenoceptor stimulation (Schmidt *et al.*, 2001). Thus, this cAMP amplification may be a more general mechanism to indirectly couple tmAC and sAC activities in bronchial epithelial cells and amplify GPCR cAMP signals. Calcium dependence of the amplification loop provides a pathway for termination of the signal via re-equilibration of $[Ca^{2+}]_i$.

The role of sAC was confirmed by both KH7 sensitivity and by shRNA-mediated reduction in CFTR activity. Several shRNA sequences were examined and one targeted to exon 15 significantly reduced the H_2O_2 response. sAC is alternatively spliced in airway epithelia (Chen *et al.*, 2014), but the exact forms playing a role here are not known. However, the lack of effect using exon 2-targeted sequence may reflect an ineffective shRNA sequence rather than presence or absence of this exon in transcripts. Despite these data supporting a sAC role, the CFTR activity was not sensitive to removal of HCO_3^- in the presence or absence of carbonic anhydrase inhibitor suggesting that sAC regulation by HCO_3^- is not fully understood.

The initial observations that inhibitors of both tmAC and sAC inhibited H_2O_2-mediated anion secretion were expected as H_2O_2 was shown previously to stimulate both EP_1 and EP_4 receptors (Jones *et al.*, 2012; Conner *et al.*, 2013) that act through Ca^{2+} and cAMP respectively. On the other hand, it was surprising that both inhibitors were able to block nearly all H_2O_2 stimulation and that both inhibitors substantially blocked EP_4 receptor stimulation that is believed to work through Gαs activation of tmAC. The observations that an Epac inhibitor was able to nearly abolish the H_2O_2 response and to block the EP_4 receptor-meidated response were equally unexpected as CFTR is activated by PKA phosphorylation. The model suggested in Figure 7 can account for the dual sAC and tmAC dependence of the CFTR response as the initial cAMP generated by Gαs stimulation would be necessary to initiate the activation of sAC that contributes a large fraction of cAMP for PKA phosphorylation of CFTR. Consistent with this idea, inhibition of either PLC or the IP_3 receptor to interrupt the Ca^{2+} signal blocked nearly all of the EP_4 receptor-mediated and H_2O_2 responses. PLC is a multifunctional family of lipases and PLCε is known to be stimulated downstream of Epac (Schmidt *et al.*, 2001) consistent with the data presented here. Together, these data suggested that an amplification mechanism was at work that included Epac activity and PLC-driven increases in $[Ca^{2+}]_i$.

It is known that Gαs-coupled receptors can increase intracellular Ca^{2+} through a variety of mechanisms in addition to Epac. For example, PG signalling has been shown to be coupled through Gβγ (Speirs *et al.*, 2010), but Gβγ did not appear to mediate H_2O_2 stimulation as it was unaffected by gallein. Stimulation of the EP_4 receptor was previously shown to activate Epac in rat neointimal formation (Yokoyama *et al.*, 2008), and an Epac inhibitor supported a role for Epac in increasing $[Ca^{2+}]_i$ that results in stimulation of both sAC and tmAC and increased activation of CFTR. Recently, the action of the Epac inhibitor ESI-09 was suggested to be due to a non-specific thermal instability (Rehmann, 2013). The IC_{50}

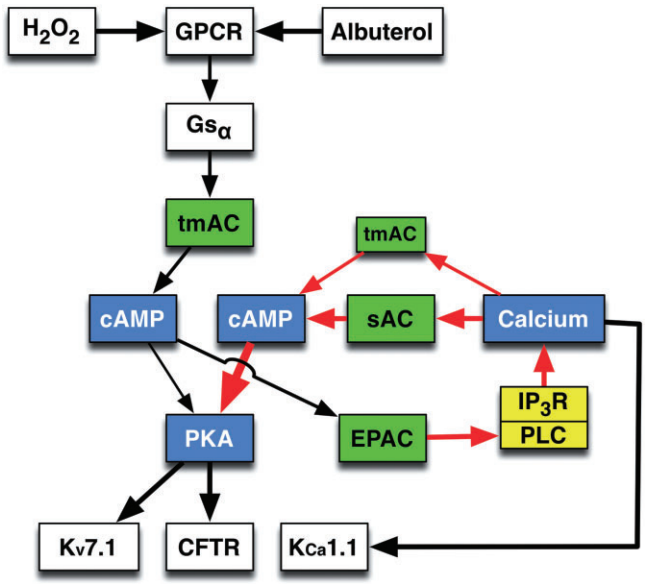

Figure 7

Proposed model for H_2O_2 stimulation of CFTR. In differentiated NHBE cells, H_2O_2 activates the GPCRs EP1 and EP4. EP4, that appears responsible for the majority of the H_2O_2 response (Conner *et al.*, 2013), and albuterol both activate Gαs to stimulate tmAC. Increased cAMP activates PKA to stimulate CFTR-mediated Cl^- secretion and basolateral $K_v7.1$ K^+ secretion. Also, cAMP activation of Epac initiates a Ca^{2+} signal presumably through PLC stimulation. This Epac-dependent Ca^{2+} signal stimulates both tmAC and sAC to amplify the cAMP/PKA pathway.

for ESI-09 effects on H_2O_2 signalling was 0.8 μM well below the ESI-09 concentration (50–100 μM) demonstrated to have induced thermal instability.

Epinephrine stimulation of β-adrenergic receptor was previously shown to activate a sAC-dependent K^+, but not Cl^-, secretion pathway in colon epithelium (Halm *et al.*, 2010). This sAC-dependent pathway affected basolateral K^+ channels; however, basolaterally permeabilized bronchial epithelial cells retained KH7 sensitivity of H_2O_2 stimulation suggesting that basolateral $K_V7.1$ found in bronchial epithelia was not the sAC-sensitive component of H_2O_2-stimulated anion currents. Conversely, H_2O_2-stimulated CFTR currents lost sensitivity to tmAC inhibitor after basolateral permeabilization suggesting that tmAC was perhaps responsible for regulating basolateral K^+ secretion after H_2O_2 stimulation.

Clearly, regulation of $[Ca^{2+}]_i$ is complex. For example, in addition to Epac regulation of $[Ca^{2+}]_i$, H_2O_2 might also alter either plasma membrane or ER Ca^{2+} ATPases or the ryanodine receptor (RyR) that is expressed in airway epithelia. It is possible that H_2O_2 stimulates anion secretion through other pathways in addition to Ca^{2+} activation of sAC. H_2O_2 is known to inhibit phosphotyrosine phosphatases and to potentiate tyrosine kinase activities that are known to stimulate CFTR (Billet and Hanrahan, 2013; Billet *et al.*, 2013), and H_2O_2 can also stimulate $K_{Ca}1.1$ channels (Liu *et al.*, 2009) to increase the driving force for Cl^- exit. In addition, others have speculated that H_2O_2 directly activates sAC in sperm during fertilization (Rivlin *et al.*, 2004). Thus, although the data presented here support the idea that H_2O_2 stimulates sAC through EP_4 receptor activation and increased $[Ca^{2+}]_i$, we cannot rule out that H_2O_2 is additionally stimulating anion secretion through other pathways.

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Author contributions

P. I. collected the research data, M. S. and G. E. C. were responsible for the research design, and all the authors contributed to the writing of the paper.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Fully differentiated NHBE cells in ALI culture were mounted in Ussing chambers and stimulated with 400 μM H_2O_2 (example trace in panel a). Panel b, H_2O_2 (400 μM) stimulation of anion currents after preincubation and in the presence of various concentrations of the the sAC inhibitor KH7 showed an apparent IC_{50} = 1.4 μM (n = 1–3 lung donors at each concentration).

Figure S2 Total RNA was extracted from fully differentiated NHBE cells in ALI culture derived from two lung donors (E.Z.N.A Omega Bio-Tek, Norcross, GA, USA), reverse transcribed (iScript cDNA synthesis kit; Bio-Rad, Hercules, CA, USA) and assayed for presence of Epac1 (TaqMan assay Hs00183449_m1), MUC5AC (TaqMan assay Hs01365601 _m1) and GAPDH as a reference mRNA (TaqMan assay 4352934E). The data demonstrate expression of Epac1 at a level slightly less than the differentiation product Muc5AC.

Figure S3 Fully differentiated NHBE cells in ALI culture were mounted in Ussing chambers and stimulated with 10 μM ATP. The trace shows an initial abrupt I_{SC} peak thought to be primarily Ca^{2+} activated Cl^- current and a later, more gradual response believed to be a sum of CFTR and Ca^{2+} activated Cl^- currents (e.g. Namkung *et al*., 2010).