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[HCO₃⁻]-regulated expression and activity of soluble adenylyl cyclase in corneal endothelial and Calu-3 cells

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Abstract

Background: Bicarbonate activated Soluble Adenylyl Cyclase (sAC) is a unique cytoplasmic and nuclear signaling mechanism for the generation of cAMP. HCO₃⁻ activates sAC in bovine corneal endothelial cells (BCECs), increasing [cAMP] and stimulating PKA, leading to phosphorylation of the cystic fibrosis transmembrane-conductance regulator (CFTR) and increased apical Cl⁻ permeability. Here, we examined whether HCO₃⁻ may also regulate the expression of sAC and thereby affect the production of cAMP upon activation by HCO₃⁻ and the stimulation of CFTR in BCECs.

Results: RT-competitive PCR indicated that sAC mRNA expression in BCECs is dependent on [HCO₃⁻] and incubation time in HCO₃⁻. Immunoblots showed that 10 and 40 mM HCO₃⁻ increased sAC protein expression by 45% and 87%, respectively, relative to cells cultured in the absence of HCO₃⁻. Furthermore, 40 mM HCO₃⁻ up-regulated sAC protein expression in Calu-3 cells by 93%. On the other hand, sAC expression in BCECs and Calu-3 cells was unaffected by changes in bath pH or osmolarity. Interestingly, BCECs pre-treated with 10 μM adenosine or 10 μM forskolin, which increase cAMP levels, showed decreased sAC mRNA expression by 20% and 30%, respectively. Intracellular cAMP production by sAC paralleled the time and [HCO₃⁻]-dependent expression of sAC. Bicarbonate-induced apical Cl⁻ permeability increased by 78% (P < 0.01) in BCECs cultured in HCO₃⁻. However for cells cultured in the absence of HCO₃⁻, apical Cl⁻ permeability increased by only 10.3% (P > 0.05).

Conclusion: HCO₃⁻ not only directly activates sAC, but also up-regulates the expression of sAC. These results suggest that active cellular uptake of HCO₃⁻ can contribute to the basal level of cellular cAMP in tissues that express sAC.

Background

Soluble adenylyl cyclase (sAC) has recently been characterized as a unique means to generate the ubiquitous signaling molecule, cyclic adenosine 3', 5'-monophosphate (cAMP) [1-3]. Elucidation of the properties of sAC indicate that this enzyme is biochemically and chromatographically different from the transmembrane adenylyl

cyclases (tmACs) [4,5]. Unlike the tmACs, sAC activity depends on the divalent cation Mn²⁺ [1,3,6], is insensitive to G protein regulation and forskolin [2,4], and displays approximately 10-fold lower affinity for ATP (K_m ≈ 1 mM) [4] than the tmACs (K_m ≈ 100 μM) [7]. HCO₃⁻ directly binds to and activates sAC in a pH-independent manner [1]. HCO₃⁻ is the primary physiological activator of sAC

and a recent study has shown that this can be modulated by Ca^{2+} [8].

Immunocytochemistry studies have demonstrated that sAC is distributed in specific subcellular compartments: mitochondria, centrioles, mitotic spindles, and nuclei, all of which contain cAMP-dependent targets [9]. Distribution at these intracellular sites indicates that sACs are in close proximity to all cAMP effectors, suggesting a model in which local concentrations of cAMP are regulated by individual adenylyl cyclases [9]. Therefore, sAC can activate many potential targets. For many years, models describing cAMP signaling in mammalian cells relied on tmAC-dependent generation of cAMP. Activation of PKA, which is anchored at various places in the cell by the scaffolding protein A kinase-anchoring protein (AKAP) [10-12], required cAMP to diffuse through the cytoplasm to propagate its signal. The elucidation of sAC removes the membrane-proximal limitation on cAMP generation and reveals new aspects of what was previously thought to be a very well characterized signaling pathway.

sAC is the predominant form of adenylyl cyclase in mammalian sperm [1-3]. The direct activation of sAC by bicarbonate in sperm provides a mechanism for generating the cAMP required for fertilization, including hyperactivated motility, capacitation, and the acrosome reaction [13-15]. sAC is also widely expressed in various tissues at low level [14]. Since bicarbonate is almost always present *in vivo*, sAC may function as a general bicarbonate/ CO_2 sensor throughout the body [15]. Furthermore, regulation of basal levels of cAMP will be influenced by active bicarbonate transport systems in those cells that express sAC. More recently, we have shown that BCECs, which express robust HCO_3^- transport activity [16,17] due to the presence of the $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC1) [17], also express sAC [18,19]. The presence of bicarbonate increases the steady-state $[\text{cAMP}]_i$, leading to phosphorylation of the apical CFTR and increased apical Cl^- permeability [18], which is an important component of the secretory function of corneal endothelial cells.

In the current study, we asked whether sAC expression can be influenced by its primary ligand, HCO_3^- . We used BCECs to show that HCO_3^- not only directly activates sAC, but also up-regulates the expression of sAC, generating a higher level of cAMP. We also examined the airway surface epithelial cell line, Calu-3, which we have also found to express sAC. These results could have relevance to many other tissues where sAC is expressed.

Results

$[\text{HCO}_3^-]$ - and time-dependent expression of sAC

Our previous studies have shown that sAC is expressed in BCEC and distributed throughout the cytoplasm [18].

Bicarbonate directly activates sAC to increase $[\text{cAMP}]_i$, which increases the phosphorylation of CFTR via cAMP-dependent PKA [18]. In the current study, we tested whether HCO_3^- can regulate the expression of sAC. RT-competitive PCR was performed to test the effect of HCO_3^- on sAC mRNA expression using specific sAC primers and internal standard GAPDH primers. First, we tested the regulation between $[\text{HCO}_3^-]$ and sAC mRNA expression. As shown in figure 1A, sAC mRNA expression in BCECs increases with increasing $[\text{HCO}_3^-]$ up to 40 mM. The decrease in the density of the GAPDH band is indicative of the competitive PCR format. The ratios of band densities (sAC/GAPDH) are summarized in figure 1B. Since 40 mM HCO_3^- maximally increased sAC mRNA expression, we chose this concentration to test sAC mRNA expression with different incubation times. As shown in figure 1C, sAC mRNA expression is significantly increased from 0 to 12 hour incubation time with no further increase at 24 or 48 hours. These results are summarized in Figure 1D.

To test whether up-regulated sAC mRNA can induce an increase in sAC protein expression, immunoblots were performed using a specific mouse anti-human sAC monoclonal antibody [9]. Both BCECs and calu-3 cells were tested. Calu-3 cells are a mixed phenotype of human bronchial epithelial and glandular cells often used to study cAMP dependent CFTR function [20,21]. RT-PCR demonstrated that sAC mRNA is also expressed in Calu-3 cells (data not shown). Figure 2 shows western blots using this antibody. For both cell types, bands at the expected 50 kD were found; some minor bands were seen with the cultured BCECs, but only a single band was seen from the calu-3 cells. sAC protein expression (figure 2A) in BCECs was increased by 45% and 87% in 10 and 40 mM HCO_3^- relative to 0 HCO_3^- , respectively. As shown in figure 2B, sAC protein expression was increased by 93% in 40 mM HCO_3^- relative to 0 HCO_3^- in Calu-3 cells, which is consistent with the results in BCECs.

The effects of extracellular pH and intracellular $[\text{cAMP}]_i$ on sAC expression

Changes in extracellular pH can regulate gene expression in renal cells *in vitro* [22]. Since culture medium pH varies with different $[\text{HCO}_3^-]$, the up-regulation of sAC expression could be induced by changes in intracellular pH (pH_i) secondary to differences in bath pH rather than HCO_3^- . The bath pH at different $[\text{HCO}_3^-]$ was determined empirically. The bath pH ranges from 6.8 at 0 HCO_3^- to 7.8 at 80 HCO_3^- , if the culture medium contains 25 mM Hepes. A full pH unit difference in bath pH causes ~0.45 pH unit change in pH_i [18]. In figure 3A, bath pH ranging from 7.0 to 8.0 did not change the expression of sAC mRNA in BCECs cultured in the absence of HCO_3^- . The ratios of competitive PCR product band densities (sAC/GAPDH) are summarized in Figure 3D. To examine sAC

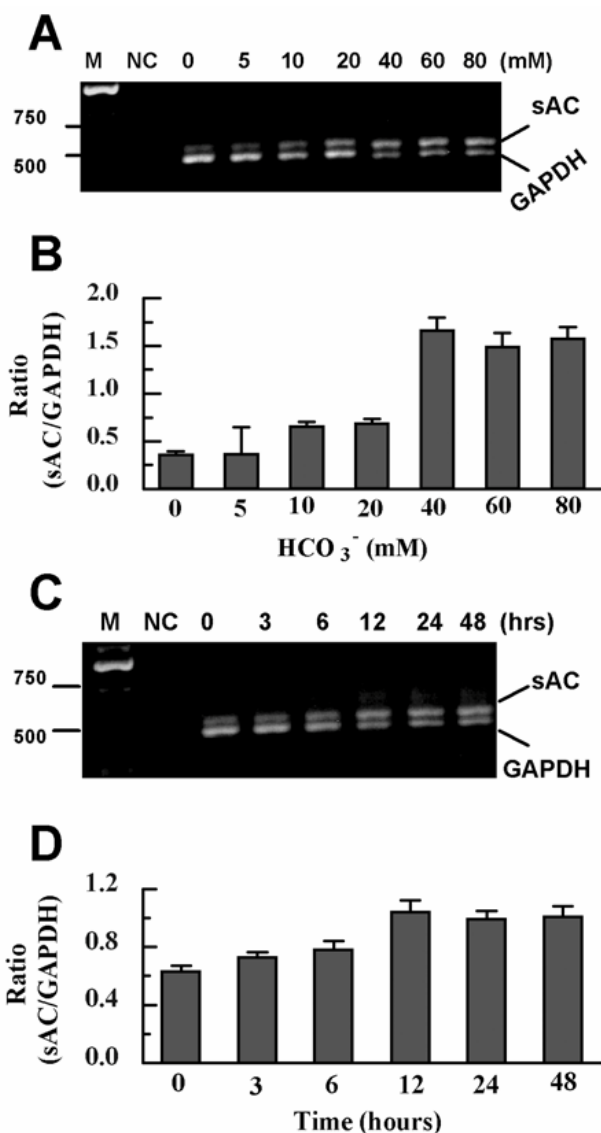


Figure 1
HCO₃⁻-induced up-regulation of sAC mRNA expression. RT-competitive PCR was performed using specific sAC primers and GAPDH internal standard primers from cultured BCECs. **A:** RT-competitive PCR of sAC mRNA from cultured BCECs exposed to different [HCO₃⁻]. From left to right: M: Marker; NC: negative control (without cDNA); [HCO₃⁻]: 0, 5, 10, 20, 40, 60 and 80 (mM). **B:** summary of the ratios of band densities (sAC/GAPDH) from A. Error bar indicates \pm SE (n = 3). **C:** RT-competitive PCR of sAC mRNA from cultured BCECs exposed to 40 mM HCO₃⁻ with different incubation time. From left to right: M: Marker; NC: negative control (without cDNA); Time: 0, 3, 6, 12, 24, 48 (hours). **D:** summary of the ratios of band densities (sAC/GAPDH) from C. Error bar indicates \pm SE (n = 3).

protein expression in different bath pH, sAC immunoblots were employed using Calu-3 cells. The effect of varying culture medium pH on sAC protein expression in Calu-3 cells is shown in figure 3B. From pH 7.0 to 8.0, equal density of sAC immunoblot bands were observed, which is consistent with the results shown in Fig. 3A.

Gene transcription regulated by cAMP, is mainly by PKA-dependent phosphorylation of the cyclic AMP response element binding (CREB) family of transcription factors (CREB, CREM, and ATF-1) [23,24]. CREB is expressed in corneal endothelium [25], so it is possible that the addition of HCO₃⁻ induces basal levels of sAC to produce cAMP, which in turn upregulates further sAC expression. Therefore, we asked whether changes in intracellular cAMP can regulate the sAC expression in BCECs. In BCECs, 10 μ M adenosine can increase [cAMP] by \sim 50%, while forskolin produces a 6–8 fold increase in cAMP [26]. The increase in cAMP induced by adenosine is similar to the increase in cAMP induced by HCO₃⁻. BCECs were treated with 10 μ M forskolin or 10 μ M adenosine for 24 hours in the absence of bicarbonate. As shown in figure 3C, the expression of sAC mRNA was decreased in adenosine and forskolin treated cells relative to the control. The ratios of band densities (sAC/GAPDH) are summarized in Figure 3D. These results suggest that the increase in [cAMP] in cells bathed in HCO₃⁻ rich solutions is not responsible for increasing sAC expression.

The effect of bath osmolarity on sAC expression

Since the osmolarity is increased with increasing NaHCO₃ concentration, it is possible that regulation of sAC expression is caused by changing bath osmolarity rather than HCO₃⁻. To exclude this possibility, we tested the effect of osmolarity alone (sucrose addition) or equimolar NaCl addition on the sAC expression. As shown in figure 4A,4B,4C,4D, the sAC expression was unaffected by increases of solution osmolarity alone or increased [NaCl], consistent with upregulation of sAC expression by HCO₃⁻.

The effect of HCO₃⁻ induced sAC expression on intracellular [cAMP]

If bicarbonate induces the up-regulation of sAC expression in BCECs, [cAMP]_i should also increase with time in bicarbonate culture medium. BCECs were HCO₃⁻-starved for 48 hours and then placed in 40 mM HCO₃⁻. As shown in figure 5A, At 0 hour, the [cAMP]_i was 19.96 ± 1.22 pmol/mg protein (n = 6). At 12 hours, [cAMP]_i was significantly increased to 34.79 ± 3.06 pmol/mg protein (n = 6). At 24 and 48 hours, however, [cAMP]_i was 33.87 ± 3.73 (n = 6) and 36.47 ± 4.50 (n = 6) pmol/mg protein respectively, which was not significantly different relative to 12 hour incubation. Therefore, these results indicate that sAC activity reached a maximal level at 12 hours in 40 mM

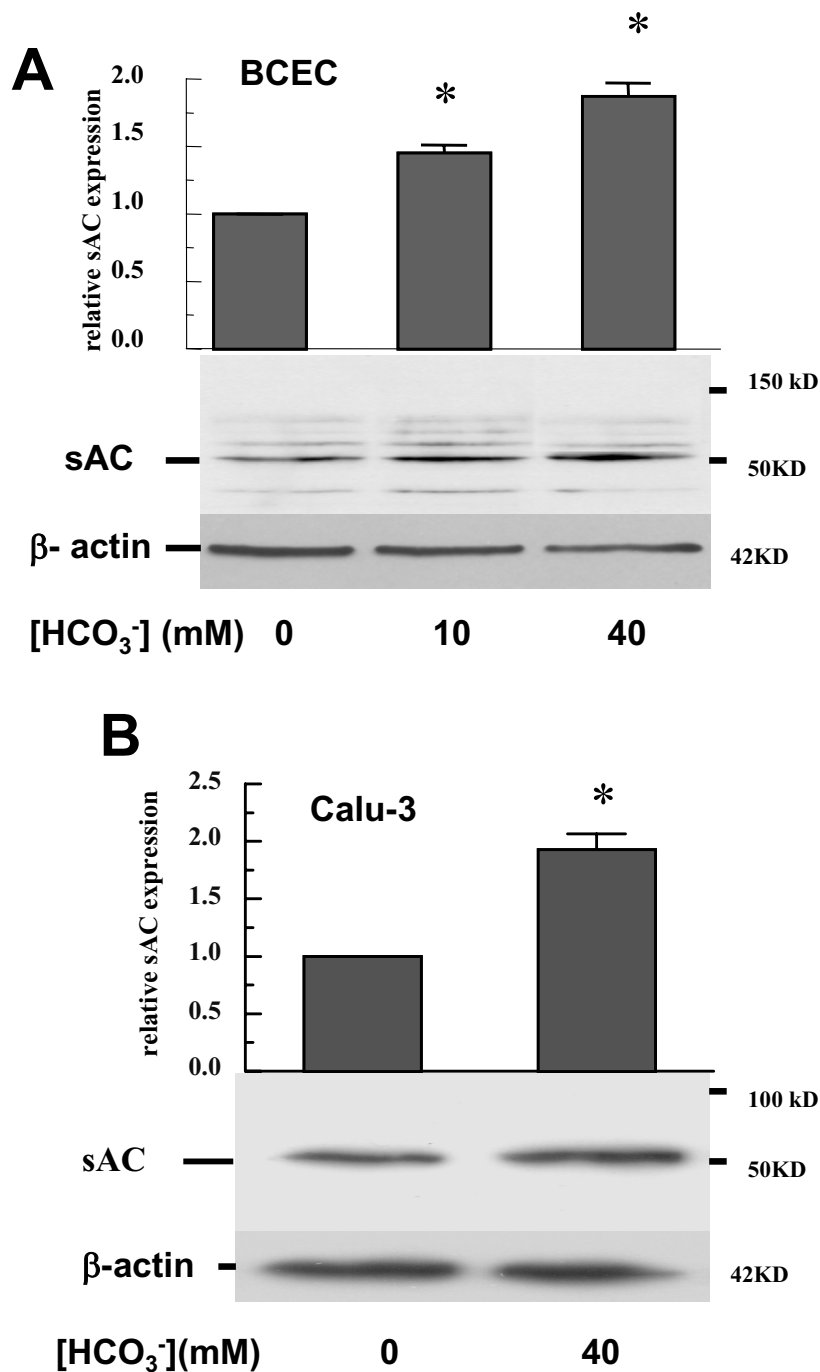


Figure 2
HCO₃⁻-induced up-regulation of sAC protein expression. Cells were starved for HCO₃⁻ in HCO₃⁻-free DMEM for 48 hours at 37°C, followed by 24-hour incubation in DMEM with different [HCO₃⁻] at 37°C. Cell lysates (60 µg/lane) were separated and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was probed with mouse anti-human monoclonal sAC primary antibody, then rinsed and reprobbed using mouse anti-human β-actin antibody. **A:** blots of sAC protein expression and β-actin from BCECs exposed to 0, 10 or 40 mM HCO₃⁻. Band density of sAC expression shown in the bar graph is relative to 0 HCO₃⁻. **B:** blots of sAC protein expression and β-actin from Calu-3 cells exposed to 0 and 40 mM HCO₃⁻. Band density of sAC expression shown in the bar graph is relative to 0 HCO₃⁻. Error bar indicates ± SE. *different from 0 HCO₃⁻ (n = 3, p < 0.05).

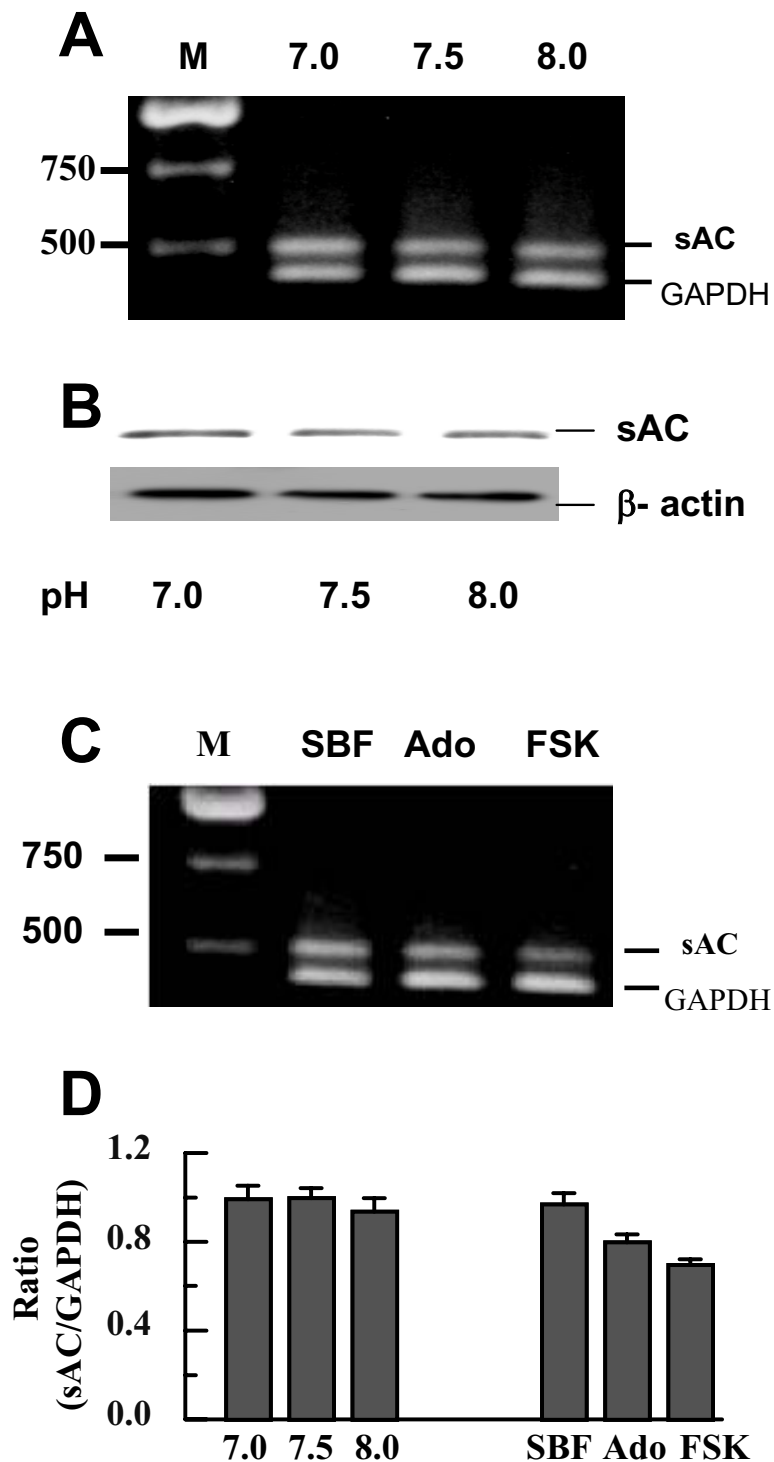


Figure 3

The effects of bath pH and intracellular [cAMP] on sAC expression. A: RT-competitive PCR of sAC mRNA from cultured BCECs exposed to different extracellular pH. From left to right: M: Marker; pH: 7.0, 7.5 and 8.0. B: immunoblots of sAC protein expression and β -actin from Calu-3 cells exposed to HCO_3^- -free culture medium at pH 7.0, 7.5 or 8.0. C: RT-competitive PCR of sAC mRNA from cultured BCECs exposed to 10 μM adenosine or 10 μM forskolin in the absence of bicarbonate. From left to right: M: Marker; SBF: standard bicarbonate free media; Ado: adenosine; FSK: forskolin. D: summary of the ratios of band densities (sAC/GAPDH) from A and C. Error bar indicates \pm SE (n = 3).

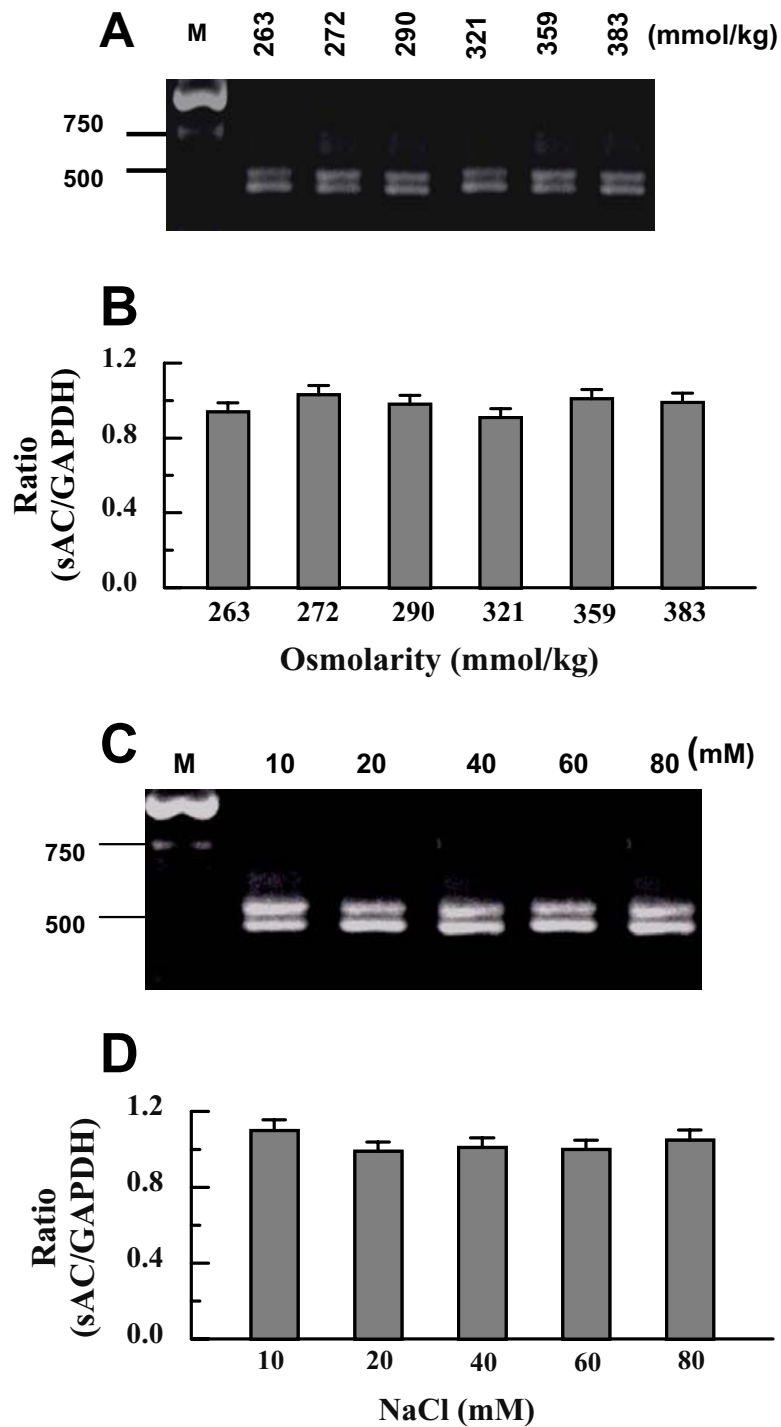


Figure 4

The effects of bath osmolarity and additional [NaCl] on sAC expression. RT-competitive PCR was performed using specific sAC primers and GAPDH internal standard primers from cultured BCECs. **A:** RT-competitive PCR of sAC mRNA from cultured BCECs exposed to different osmolarity (sucrose added). From left to right: M: Marker; osmolarity: 263, 272, 290, 321, 359, 383 (mmol/kg). **B:** summary of the ratios of band densities (sAC/GAPDH) from A. Error bar indicates \pm SE (n = 3). **C:** RT-competitive PCR of sAC mRNA from cultured BCECs exposed to different [NaCl]. From left to right: M: Marker; [NaCl]: 10, 20, 40, 60, 80 (mM). **D:** summary of the ratios of band densities (sAC/GAPDH) from C. Error bar indicates \pm SE (n = 3).

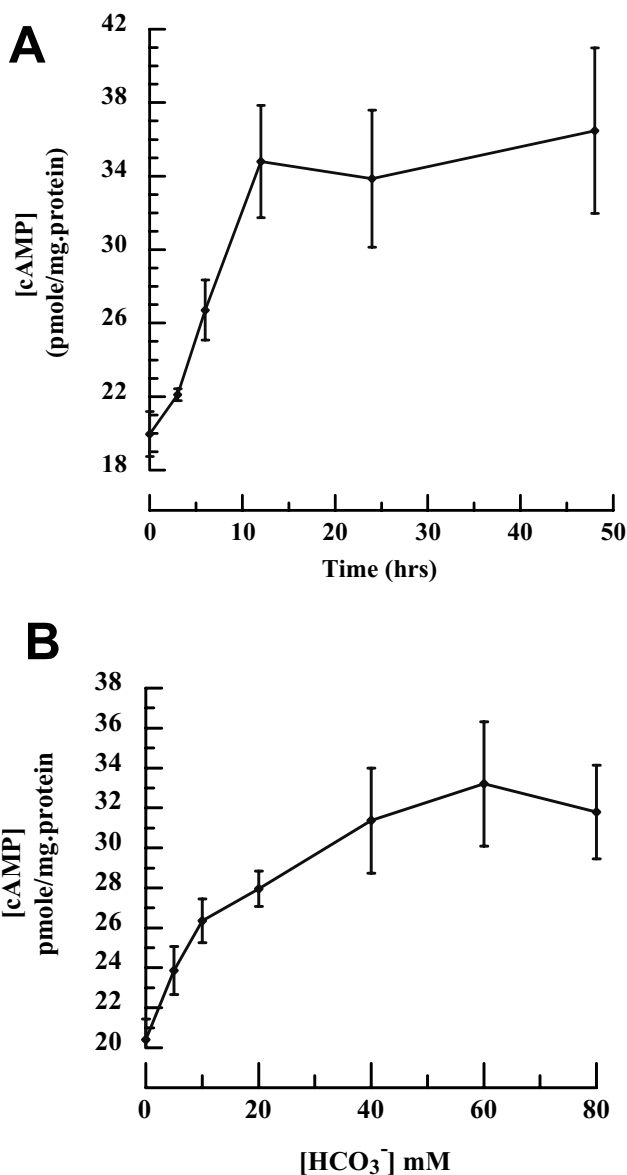


Figure 5
The effects of HCO₃⁻-regulated sAC expression on intracellular cAMP accumulation. Culture medium was removed from confluent cultured BCECs and replaced with HCO₃⁻-free DMEM for 48 hours at 37°C. Cells were then incubated either for different time in 40 mM HCO₃⁻ DMEM or for 24 hours in different [HCO₃⁻] at 37°C. Cells were lysed in 0.1 N HCl. [cAMP] was measured by enzyme immunoassay. **A:** changes in intracellular [cAMP] from cells exposed to 40 mM HCO₃⁻ with 0, 3, 6, 12, 24 and 48 hour incubation. **B:** Intracellular [cAMP] from cells exposed to 0, 5, 10, 20, 40, 60 and 80 mM HCO₃⁻ for 24 hour incubation. Error bars indicate ± SE (n = 6)

HCO₃⁻, which is consistent with the maximal increase in sAC mRNA (see figure 1C). In figure 5B, BCECs were HCO₃⁻-starved for 48 hours and then placed in different [HCO₃⁻] for 24 hours. At 0 HCO₃⁻, the [cAMP]_i was 20.40 ± 1.03 pmol/mg protein (n = 6). [cAMP]_i increased with increasing [HCO₃⁻] (K_{1/2} ≈ 10 mM HCO₃⁻) and was maximal between 40 and 60 mM. Higher [HCO₃⁻] did not produce any further significant increase in [cAMP]. Taken together the results from fig. 5A and 5B are consistent with the results from HCO₃⁻-induced sAC protein and mRNA expression.

The effect of HCO₃⁻-induced sAC expression on apical Cl permeability in BCECs

Acute exposure to HCO₃⁻ activates sAC, increases [cAMP] and PKA activity, leading to the increased phosphorylation of CFTR and increased apical Cl⁻ permeability [18]. Since culturing in the absence of HCO₃⁻ can down-regulate sAC expression and intracellular [cAMP] in BCECs, acute exposure of bicarbonate-starved cells to HCO₃⁻ should show a reduced increase in apical Cl⁻ permeability relative to cells cultured in the presence of bicarbonate. To test this possibility, we loaded bicarbonate-starved BCECs with the halide-sensitive dye MEQ and measured the rate of fluorescence change in response to apical Cl⁻ pulses. Both apical and basolateral sides were initially perfused with Cl⁻ and HCO₃⁻-free solutions. As shown in figure 6A, when Cl⁻ was added to the apical side for 90s, a small, slow decrease in MEQ fluorescence, caused by the entry of Cl⁻, was observed. Both sides were then bathed in Cl⁻-free, HCO₃⁻-rich solution for at least 5 min. When the fluorescence signal had stabilized, Cl⁻ was applied to the apical side in the presence of HCO₃⁻. The decrease in MEQ fluorescence was not significantly changed relative to the paired control. Figure 6B summarizes the results and shows that the apical Cl⁻ permeability of HCO₃⁻-starved BCEC was increased by 10.3% in the presence of HCO₃⁻. However, the apical Cl⁻ permeability in BCECs cultured in bicarbonate, as shown in figure 6C, was significantly increased relative to the paired control. Figure 6D summarizes the results and shows that the apical Cl⁻ permeability of BCECs cultured in bicarbonate was increased by 78% in the presence of HCO₃⁻.

Discussion

This study is the first demonstration that bicarbonate up-regulates sAC expression in a dose- and time-dependent manner. In BCECs, RT-competitive PCR identified that sAC mRNA expression is significantly increased with increasing [HCO₃⁻] or incubation time in HCO₃⁻. Western blot gave a major band with BCECs at the expected size, which was confirmed using the human calu-3 cells. Furthermore, sAC protein expression is increased by incubation in HCO₃⁻ in both BCECs and Calu-3 cells.

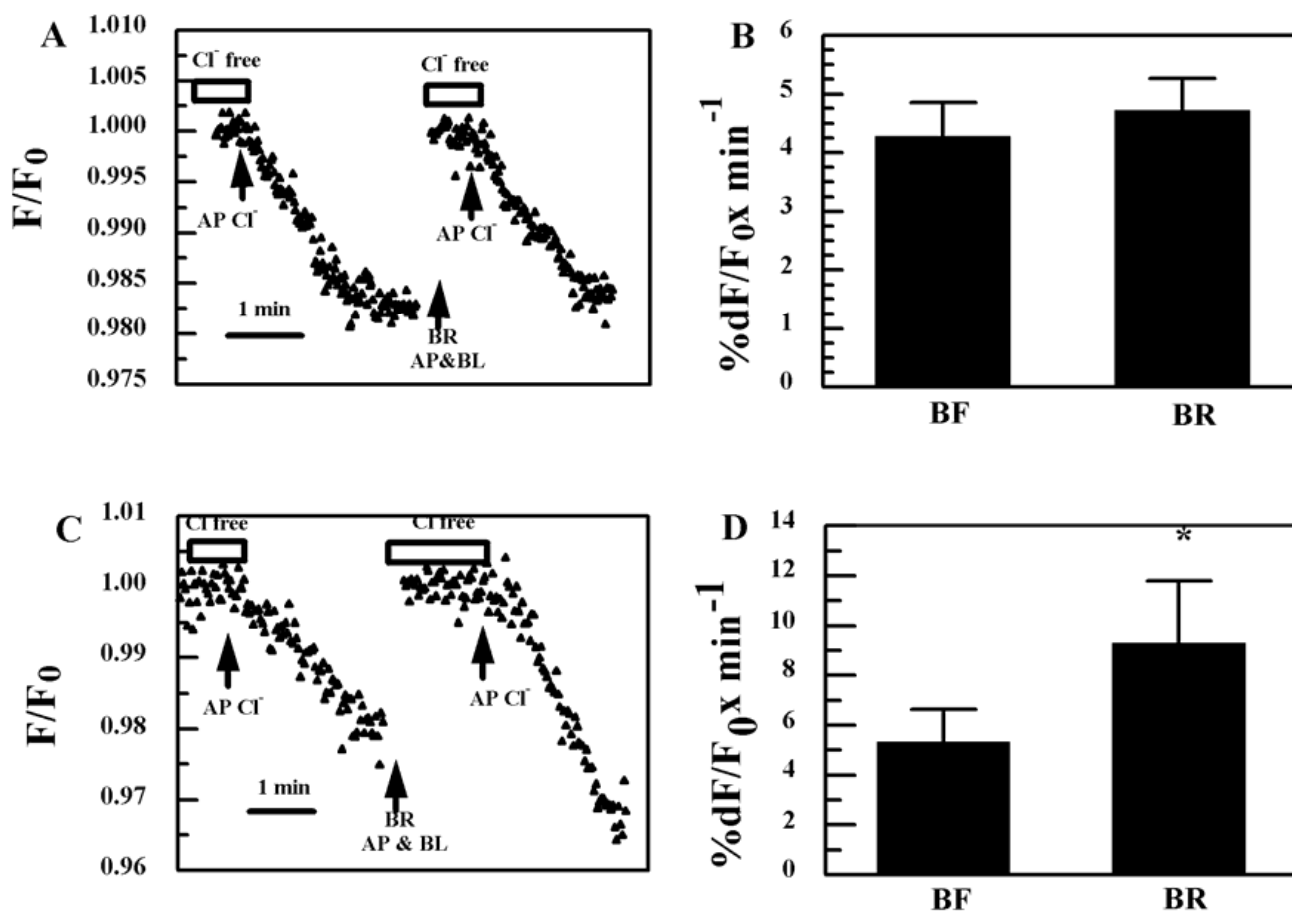


Figure 6

The effect of HCO_3^- -induced sAC expression on apical Cl^- permeability in BCECs A: HCO_3^- -starved BCECs grown on permeable Anodisc. Both apical (AP) and basolateral (BL) compartments were initially perfused with Cl^- - and HCO_3^- -free Ringer solution. After the 1st apical (AP) Cl^- pulse, HCO_3^- -rich Ringer solution (BR) was introduced on both sides for at least 5 min before the 2nd Cl^- pulse. Break in trace indicates period of wash in Cl^- -free and BR solution until trace stabilized (at least 5 min). B: maximum slope summary data for A ($n = 5$); all fluorescence values were normalized to the fluorescence value in the absence of Cl^- (F_0) obtained just before addition of Cl^- . Calculated slopes were adjusted by any background drift in the fluorescence trace that was apparent just before addition of Cl^- . C: BCECs cultured in BR medium, same experiment as in A. D: summary data for C ($n = 5$). *Significantly different from HCO_3^- -free solution (BF) ($P < 0.05$).

Since bath pH is changed at different $[\text{HCO}_3^-]$, sAC expression could be induced by pH rather than HCO_3^- . Previous studies have shown that pH changes can be a regulatory factor for stabilization of some mRNA [27]. Direct regulation of mRNA stability by pH requires that the 3'-untranslated region of the regulated mRNA contain a direct repeat of an eight-base AU sequence that functions as a pH-response element [27]. This sequence increases the binding of ζ -crystallin/NADPH:quinine reductase, which may initiate the pH-responsive stabilization of the regulated mRNA [27]. Therefore, in Fig. 3A and 3B, we tested whether varying bath pH may induce sAC mRNA and protein expression in BCECs and Calu-3 cells. Bath pH from

7.0 to 8.0 had no effect on sAC expression. This finding is consistent with the fact that sAC does not contain an eight-base AU repeat in the 3'-untranslated region [2,4].

sAC activity is not only modulated by bicarbonate, but also by calcium [8]. Therefore cytosolic $[\text{Ca}^{2+}]$ could also be a possible regulatory factor of sAC expression. In some cell types, changes in external pH can alter cytosolic $[\text{Ca}^{2+}]$. However, the pH-independence of sAC expression shown here (figure 3A and 3B) excludes this possibility. Furthermore, we have previously shown that changing perfusion of cultured BCEC between bicarbonate-free to bicarbonate-rich conditions has no effect on $[\text{Ca}^{2+}]_i$ [28].

However, this does not exclude the possibility that altering $[Ca^{2+}]_i$ by some other means could affect sAC expression.

Changing $[NaHCO_3]$ between 0 and 80 mM significantly affects medium osmolarity, which could be responsible for the change in sAC expression. However, decreasing or increasing osmolarity or increasing $[NaCl]$ had no effect. Moreover, from figure 1A, the biggest change in sAC expression is between 20 mM and 40 mM HCO_3^- . However, the osmolarity change caused by this increase in $NaHCO_3$ is only 18 mmol/kg (from 299–317 mmol/kg). Much larger changes in osmolarity (e.g., 40 to 80 mM $NaHCO_3$) had no effect on sAC expression.

Bicarbonate-regulated sAC represents an alternate source for cAMP [1-3,18]. sAC is not solely a soluble protein but is specifically targeted to well-defined intracellular compartments – mitochondria, centrioles, mitotic spindles and nuclei – in close proximity to intracellular effectors of cAMP signaling [9]. As shown in Fig. 3C, either forskolin or adenosine can significantly decrease sAC expression compared to bicarbonate-free control, suggesting that higher $[cAMP]_i$ down-regulates the expression of sAC. These observations indicate that HCO_3^- -induced up-regulation of sAC is not caused by increasing $[cAMP]$. Conversely, these results are consistent with the notion that sAC contributes to regulating basal levels of cAMP. Possibly, when cAMP levels increase above some basal set-point, sAC is down-regulated. This raises the following questions: 1) Is cAMP-induced sAC down-regulation caused by phosphorylated CREB? 2) Would activated CREB decrease the transcription of sAC mRNA? 3) How does HCO_3^- increase sAC mRNA level? sAC can be found in the nucleus [9], thus possibly sAC itself is associated with a transcription factor that is activated upon binding by HCO_3^- .

As we demonstrated previously [18], activated sAC in BCECs can stimulate CFTR via up-regulating the intracellular $[cAMP]$ and increasing PKA activity, leading to increased phosphorylation of CFTR and thus increased apical Cl^- permeability. In the current study, we used HCO_3^- -starved BCECs to test the effect of down-regulated sAC on the apical Cl^- permeability. Due to the basal levels of sAC expression in HCO_3^- -starved BCECs (as shown in figure 2A and 2B), HCO_3^- produced a small (10.3%), but not significant, increase in apical Cl^- permeability compared to the control (fig 6B). It is possible that some cAMP is likely being produced, but it is also being quickly degraded by the cAMP specific phosphodiesterase, PDE4 [18]. Thus the basal amount of sAC is not sufficient to raise the steady-state level of $[cAMP]$ such that CFTR permeability is affected. However, the HCO_3^- stimulated apical Cl^- permeability in BCECs cultured in HCO_3^- was

significantly increased relative to the control. This finding not only is consistent with our previous report [18], but also confirms that HCO_3^- -regulated sAC expression affects the basal level of apical Cl^- permeability in BCECs.

Conclusions

In the current study, we identified that HCO_3^- can up-regulate functional sAC. Coupled with our recent study that HCO_3^- -activated sAC in BCECs can generate cAMP leading to phosphorylation of apical CFTR, which significantly enhances apical chloride permeability, this novel finding has important implications for mammalian cells such as corneal endothelium and Calu-3 cells. In corneal endothelial cells, chloride and bicarbonate are essential for transendothelial fluid transport to maintain corneal transparency [30-33]. Chloride and bicarbonate secretion at the apical membrane (aqueous humor side) is stimulated by increasing cytosolic $[cAMP]$ [34-36]. Furthermore, cAMP activates chloride transport in cultured corneal endothelial cells [37,38] and cAMP-dependent CFTR significantly contributes to apical Cl^- and HCO_3^- efflux in corneal endothelium [38]. Therefore, HCO_3^- -regulated sAC expression may play a pivotal role in maintaining steady-state anion transport and fluid secretion by the corneal endothelium. In addition, Calu-3 cells share many of the same anion transport mechanisms as corneal endothelium. Thus the presence of HCO_3^- and sAC need to be considered when evaluating basal cAMP dependent anion transport in Calu-3 cells.

Methods

Cells

Fresh cow eyes were obtained from a local slaughterhouse and kept on ice until use, ~4 hours after death. Primary cultures of bovine corneal endothelial cells (BCEC) were grown to confluence as previously described [39,40]. Calu-3 cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

Analysis of sAC mRNA expression by competitive RT-PCR

A pair of human sAC primers was constructed on the basis of the published cDNA sequence [2]. The sAC sense primer was 5'-CCTGGAATAACCTGTTCAAG-3' and the sAC antisense primer was 5'-TCTGGTCCITGAGCCACAG-3'. The expected length of PCR products for sAC was 544 bp. Another pair of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers was constructed on the basis of the published bovine cDNA sequence (GenBank access no. U85042). The GAPDH sense primer was 5'-TGACCCCTTCATTGACCTTC-3' and the antisense primer was 5'-GGTCATAAGTCCCTCCAGGA-3'. The expected length of PCR products for GAPDH was 460 bp.

Confluent cultured BCECs in 35 mm Petri dishes were starved for bicarbonate in HCO_3^- -free DMEM for 48 hours at 37°C . Cells were then incubated either for different times in 40 mM HCO_3^- DMEM or for 24 hours in varying concentrations of HCO_3^- . HCO_3^- -free DMEM was purchased from GIBCO (Carlsbad, CA). Different concentrations of HCO_3^- DMEM were prepared by adding varying amounts of NaHCO_3 . Different osmolarity DMEM solutions were adjusted using sucrose to be equivalent to the osmolarities of the different [HCO_3^-] DMEM solutions. The method to prepare different [NaCl] solution is the same as preparing different [HCO_3^-] DMEM solutions except for replacing NaHCO_3 with NaCl . In addition, BCECs without bicarbonate starvation were incubated in HCO_3^- -free DMEM either at different bath pH or with 10 μM adenosine or 10 μM forskolin for 24 hours at 37°C .

Total RNA was extracted from BCECs using TRIzol reagent (Invitrogen) as previously described [17,41]. The RNA was DNase-treated using an RNase-free DNase set (Qiagen). Reverse transcription was performed using Superscript™ cDNA synthesis system (Invitrogen) and oligo (dT) primers as previously described [17,41]. Competitive PCR amplifications were carried out in a thermocycler using the high fidelity TaKaRa Ex Taq PCR system kit (TaKaRa Shuzo) under the following conditions: denaturation at 94°C for 3 min for one cycle, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension for one cycle at 72°C for 10 min. The PCR products were loaded onto 1% agarose gels, electrophoresed, and stained with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. The density of each band was digitized using Un-Scan-It software (Silk Scientific). The ratio of sAC band density to parallel GAPDH band density was compared within each experiment.

Competitive RT-PCR was used throughout this study. Thus, as sAC expression increases, the amount of GAPDH PCR product formed drops. The ratio of the two sets of primers (sAC and GAPDH) in the starting amplification reaction was varied for each type of experiment in order to assure that bands for both sAC and GAPDH could be detected. Thus comparisons can be made within each type of experiment (e.g., within figure 1 or figure 3), but not between experiments (i.e., figure 1 cannot be compared with figure 3).

PCR products were purified using a 1% low-melting point agarose gel. Freshly purified products were inserted into pCR 4-TOPO vector (Invitrogen, San Diego, CA) and sequenced as previously described [17,41]. Sequences were assembled and compared using Vector NTI version 5.2 software (InforMax, North Bethesda, MD).

Immunoblotting

Confluent cultured BCECs and Calu-3 cells were starved for bicarbonate, followed by a 24-hour incubation in different concentrations of bicarbonate in DMEM at 37°C . Calu-3 cells were also incubated in HCO_3^- -free DMEM with different pH for 24 hours. Cells were then lysed in chilled lysis buffer (150 mM NaCl , 0.5% sodium deoxycholate, 1% SDS, 50 mM Tris, pH 8.0, 1 mM EDTA, 0.1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin) and cleared by centrifugation. Cell lysates (60 $\mu\text{g}/\text{lane}$) were separated by 8% SDS-PAGE, and transferred to a polyvinylidene fluoride (PVDF) membrane, as previously described [38]. The membrane was blocked and probed with mouse anti-human sAC primary antibody (1:1000, a kind gift from Dr. J Buck and L Levin, Medical College of Cornell University) [9] and goat anti-mouse secondary antibody coupled to horseradish peroxidase (1:5000, Sigma). The same transferred membrane was rinsed and reprobed using mouse anti-human β -actin to test the loading of total protein. Exposed films were scanned and the density of equal areas of the developed bands was estimated using Un-Scan-It software (Silk Scientific).

Determination of Intracellular cAMP Accumulation

Culture medium was removed from confluent cultured BCECs and replaced with HCO_3^- -free DMEM for 48 hours at 37°C . Cells were then incubated either for different time in 40 mM HCO_3^- or for 24 hours in different concentrations of HCO_3^- at 37°C . At the end of the incubation, cells were washed with PBS and lysed in 0.1 N HCl and cleared of cellular debris by centrifugation. [cAMP] was measured by an enzyme immunoassay kit (R&D Systems) and an ELISA plate reader (Fluostar galaxy, BMG Labtechnologies).

Apical Cl^- permeability

Relative changes in apical Cl^- permeability were assessed with the halide-sensitive fluorescent dye MEQ. Confluent BCEC, grown on permeable Anodiscs and starved for HCO_3^- for 24 hours, were loaded with MEQ by exposure to diH-MEQ for 10 min [37,38] 42. Anodiscs were placed in a double-sided microscope perfusion chamber and apical and basolateral compartments were independently perfused at 37°C . MEQ fluorescence (excitation: 365 ± 10 nm; emission: 420–450 nm) was measured as previously described [37,38] 42. Anodiscs were initially perfused with a Cl^- and HCO_3^- -free solution (in mM: 150 Na^+ , 4 K^+ , 0.6 Mg^{2+} , 1.4 Ca^{2+} , 148.5 NO_3^- , 2 HPO_4^{2-} , 10 HEPES, 2 gluconate, and 5 glucose, pH 7.5) and the apical side briefly pulsed with Cl^- -rich Ringer's (equimolar replacement of 118 NaNO_3 with NaCl). HCO_3^- -Rich solutions were prepared by equimolar substitution of 28.5 NaNO_3 with NaHCO_3 , gassed with 5% CO_2 , pH 7.5. Relative changes in apical Cl^- permeability between control and experimental conditions in the same cells were

determined by comparing the percentage change in MEQ fluorescence (F/F_0) after addition of Cl^- , where F_0 is the fluorescence in the absence of Cl^- . The maximum slope of fluorescence change was determined by calculating the first derivative using Felix software (Photon Technology International).

Data analysis

All data were expressed as means \pm SE and Student's paired *t*-test was used for statistical analysis at $p < 0.05$.

Authors' contributions

XCS conceived and designed this study, performed most of the experiments and wrote the manuscript. MC performed some RT-PCR experiments and cultured the cells. JAB helped design the study and interpret the results, and revised the final version of the manuscript.

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