Regulation of CFTR channels by HCO$_3^-$-sensitive soluble adenyl cyclase in human airway epithelial cells

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CFTR IS THE ANION CHANNEL defective in cystic fibrosis (CF), a lethal hereditary disease. CFTR is subject to complex and integrated phosphorylation/dephosphorylation regulation by multiple enzymes, including PKA, PKC, Src tyrosine kinase, AMP-dependent kinase, and phosphatases. Of the kinases activating CFTR, PKA appears to be the most important pathway in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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be verified at the single-channel level. A better understanding of sAC regulation of CFTR calls for further studies with more direct and relevant measurement of CFTR activity. In addition, it is of interest to extend these studies to the airway epithelium, where CFTR and its HCO$_3^-$ transport function play a crucial role in the innate defenses of the lung. In the present work, in which we used patch-clamp techniques and a specific blocker of sAC, we studied the regulation of CFTR by sAC in Calu-3 cells, a widely used cellular model of human airway epithelia.

**MATERIALS AND METHODS**

**Cells.** Human Calu-3 cells (HTB-55; American Type Culture Collection, Manassas, VA) were grown in Eagle’s minimum essential medium (MEM; Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 10% sodium pyruvate, and penicillin/streptomycin in an atmosphere of 95% air-5% CO$_2$ at 37°C. Cells were passed at a 1:3 dilution every 5–7 days. For cAMP assay, cells were grown to confluence on clear Transwells (Costar) with a resistance >1,000 Ω·cm$^2$. For patch-clamp and other studies, cells were grown on plastic dishes and used for experiments after 3–7 days.

**RT-PCR.** A pair of sAC primers flanking the region deleted in T-sAC was constructed on the basis of the published cDNA sequence (14). The sense and antisense primers were 5'-CTGTATCCATCG-GTGT-3' and 5'-TTGTCGGTGTCGTCTTCT-3', respectively. Total RNA was extracted from Calu-3 cells with the RNeasy Mini kit (Qiagen). RT-PCR was performed with the OneStep RT-PCR kit (Qiagen). PCR was performed for initial denaturation of one cycle at 94°C for 10 min, followed by 35 cycles of denaturation (94°C for 45 s), annealing (52°C for 45 s), and extension (72°C for 45 s), and a final extension for one cycle at 72°C for 10 min. At the end of the PCR amplification, products were analyzed in 1.5% agarose gels stained with ethidium bromide and visualized under UV light. The amplified fragments of correct size were excised from the agarose gel and purified using a gel extraction kit (Qiagen). The second-round PCR was conducted using the purified PCR products as the template and another pair of sAC primers (sense primer, 5'-AGACTTGGCTGCTGTTAG-3'; antisense primer, 5'-ATTGTGACCTGCA-TTTAG-3'). PCR was performed for an initial denaturation of 1 cycle at 94°C for 4 min and 39 cycles at denaturation (94°C for 30 s), annealing (50°C for 30 s), and extension (72°C for 30 s) with Taq polymerase (Invitrogen). This was followed by a final extension step at 72°C for 10 min. PCR products of the correct size of two splice variants were excised for sequencing.

**Generation and purification of sAC antibody.** A rabbit polyclonal antisera was raised against a synthetic peptide of 17 amino acids (KEEFQDWPIVRIA-COOH). The serum was affinity-purified rabbit anti-sAC serum with or without 4 mg/ml antigen peptide or a monoclonal antibody against the CFTR COOH terminus (R&D Systems). The membrane was washed three times with TBST and incubated for 1 h with a horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:10,000 in TBST containing 5% nonfat dry milk. After being washed several times with TBST, bound antibodies were detected using enhanced chemiluminescence (Pierce).

**cAMP assay.** Cells were incubated in 25 mM HEPES-buffered, air-equilibrated, HCO$_3^-$-free MEM, pH 7.2, for 3 h at 37°C, followed by 10-min incubation with the mucosal addition of 40 mM NaHCO$_3$ or 10 μM forskolin as positive control or 40 mM NaN$_3$, as a negative control. NaHCO$_3$ solution was made in the assay buffer immediately before starting the assay and was buffered to pH 7.2 with predetermined amounts of HEPES (40 mM NaHCO$_3$ + 8 mM HEPES), and NaHCO$_3$-containing reaction mixtures were placed in an atmosphere of 95% air-5% CO$_2$. Papaverine (200 μM) and 300 μM 8-(p-sulfophenyl)theophylline (8-SPT) were present in all reactions. Papaverine is a nonspecific phosphodiesterase inhibitor, and 8-SPT was used to block the autocrine stimulation of A$_2$A adenosine receptors and thus reduce cAMP background (11). The cells were then lysed in 0.1 N HCl, and cAMP was measured using a cAMP immunoassay kit (Assay Designs).

**Whole cell voltage-clamp studies.** CFTR-mediated whole cell Cl$^-$ current was recorded as previously described previously (12). Briefly, the pipette solution contained (in mM) 40 Tris-Cl$, 100$ Tris-glucosone, 2 MgCl$_2$, 5 HEPES, 1 E GTA, and 0.1 CaCl$_2$, 1 MgATP, and 0.2 LiGTP, pH 7.4, with Tris. Ca$^{2+}$ activity was buffered to ~40 nM. The bath solution contained (in mM) 150 Tris-Cl$, 2 M$ MgCl$_2$, 1 CaCl$_2$, 5 HEPES, 30 sucrose, 10 d-glucose, pH 7.4, with Tris. Patch pipettes had a resistance of ~3 MΩ with these solutions. In a standard protocol, voltage was clamped to ~40 mV and stepped to 60 mV every 10 s as current was recorded. Current-voltage (I-V) plots were obtained every 2 min from the current responses to step pulses from ~100 to 100 mV in 20-nV increments. The whole cell conductance was calculated by the slope of a linear fit of the I-V relationship and was used as readout of CFTR channel activity as previously established (12). After the whole cell recording configuration was obtained, basal current was recorded for 6 min in the presence or absence of 20 μM 2-hydroxyestradiol (2-HE). Freshly made 25 mM NaHCO$_3$ (buffered with 5 mM HEPES) was then added to the bath, and whole cell current was recorded for 8 min. The bath solution was not gassed with 5% CO$_2$, and the pH change of the HCO$_3^-$-containing bath solution was minimal in an 8-min period (~0.02 pH units). HCO$_3^-$-stimulated slope conductance was calculated from the I-V plot with the highest slope conductance. Reversal potentials were calculated using V$_{pipet}$ at zero current with correction for the junction potential of the pipette and bath solution (~5 nM).

**Cell-attached recording.** The procedures were essentially the same as previously described (12), with some modification. The bath contained (in mM) 150 Tris-Cl$, 1 M$ CaCl$_2$, 2 MgCl$_2$, 10 mM-glucose, 5 HEPES, pH 7.4 maintained with Tris. The pipette solution contained 160 mM Tris-Cl$^-$ and 30 mM sucrose at pH 7.4 adjusted with Tris. After the formation of a cell-attached seal, CFTR single-channel activity was recorded for 5 min at V$_{pipet}$ of ~60 mV in the presence or absence of 20 μM 2-HE. Subsequently, freshly made 25 or 50 mM NaHCO$_3$ (buffered to pH 7.4 with HEPES) was added into the bath, and the activity of CFTR was recorded for 5 min. In our experiments, CFTR channels were identified by typical burstlike openings and flickery kinetics at the positive holding potential and the slope conductance of ~7.0 pS in excised patches. The identity of these channels has previously been confirmed to be CFTR on the basis of extensive work by others and by us (12, 13).

**Data acquisition and analysis of patch-clamp studies.** Data were acquired using an Axopatch 200B amplifier and Axon DigiData 1322A with Axon pClamp 9 software. Single-channel currents were filtered at 100 Hz and sampled at 10 kHz. The product of CFTR...
channel number (n) and open probability (P_o) was calculated as CFTR channel activity (nP_o). nP_o was calculated from the 100-s segment recorded before addition of NaHCO₃ or from the 100-s segment (of 300 s) with the highest nP_o after addition of NaHCO₃. Whole cell current was acquired at 500 Hz and filtered at 100 Hz. All data were analyzed using Clampfit 9.0 software.

Statistics. All of the data are expressed as means ± SE. Statistical analysis was performed with GraphPad Prism 4.0 software. Unless indicated otherwise, Student’s t-test was used for statistical analysis. P < 0.05 was considered as statistically significant.

Reagents. Oligonucleotides were obtained from Proligo (Singapore). All other reagents were obtained from Sigma unless indicated otherwise.

RESULTS

RT-PCR experiments were performed to detect the expression of the two sAC transcripts in Calu-3 cells. Initial RT-PCR trials with several different pairs of primers closely flanking the deletion region generated two PCR bands with slightly different mobility (Fig. 1A, left, results shown are of one pair of these primers), which were expected to correspond to FL-sAC and T-sAC transcripts, respectively (14). DNA sequencing confirmed that the upper band was a FL-sAC transcript but failed to identify T-sAC transcript in the lower band because of overwhelming nonspecific DNA sequences. To circumvent this problem, the lower band in the first round of RT-PCR was excised, extracted, and used as a template for another round of PCR with a different pair of primers (see MATERIALS AND METHODS). The second round of RT-PCR generated two bands (Fig. 1A, right), in which the lower band had the predicted size of the T-sAC transcript and the upper band represented the contamination of FL-sAC transcripts in the excised template. DNA sequencing confirmed the identity of the PCR products of two bands to be FL-sAC and T-sAC transcripts (Fig. 2). These findings demonstrate that there are two different transcripts of sAC in human cells as well (14).

With the use of anti-sAC antiserum, a protein migrating coincident with the T-sAC positive control mouse kidney tissue (5) was found in the whole cell lysate of Calu-3 cells (Fig. 1B). No reliable band of FL-sAC was detected in Calu-3 cells, although it was detected in the mouse testis tissue as a predominant band (Fig. 1B). The predominant presence of T-sAC in Calu-3 cells confirmed the results in a previous study (26) and is in line with observations in many other cells (4, 30). Consistent with its apparent lack of transmembrane domains, a major portion of sACs was present in the cytosol (Fig. 1C). Interestingly, a fraction of T-sAC (~7%) (Fig. 1C) were present in the particulate fraction, suggesting that sAC could be membrane associated as well.

A unique feature differentiating sAC from TMACs is HCO₃⁻ sensitivity. Exploiting this characteristic, we tested whether sAC is functional in Calu-3 cells by determining HCO₃⁻-sensitive adenylyl cyclase activity. The addition of 40 mM NaHCO₃/5% CO₂ (buffered to pH 7.2 with HEPES) resulted in a small yet highly reproducible intracellular cAMP increase (0.30 ± 0.02-fold over control, n = 5; P = 0.0008) (Fig. 3). The cAMP increase resulted from HCO₃⁻ instead of Na⁺ because adding 40 mM NaN₃O₃ had virtually no effect on cAMP increase (~0.13 ± 0.10-fold over the control, n = 3; P > 0.1). These data support the presence of functional sAC in Calu-3 cells. However, caution should be taken when interpreting the effect of HCO₃⁻ of physiological concentration, because HCO₃⁻ concentration and pH have reciprocal effects. Although NaHCO₃ solution was buffered with HEPES to avoid extracellular pH change resulting from HCO₃⁻ addition, we found that adding HCO₃⁻ solution resulted in a transient drop in intracellular pH (~0.13 to 0.20 pH units, which recovered in 2–3 min; n = 3) as measured using pH-sensitive fluorescent dye seminaphthorhodafluor-5F 5-(and-6)-carboxylic acid ace-toxymethyl ester acetate (S-23922; Molecular Probes), which, although small, could potentially contribute to the observed cAMP change by affecting TMAC activity (18). To confirm that HCO₃⁻-stimulated cAMP increase indeed resulted from
sAC, we used a sAC inhibitor, 2-HE. Braun first reported 2-HE to block sAC in 1990 (2), and this finding was recently verified by using purified recombinant rat sAC (23). However, little is known concerning its specificity and possible effect on TMACs. To clarify this issue, we evaluated the effect of 2-HE on HCO₃⁻ and forskolin-stimulated cAMP production and included a TMAC inhibitor, SQ22,536, as a control. HCO₃⁻-stimulated cAMP production was completely eliminated by 2-HE but was not affected by SQ22,536, although SQ22,536 partially reduced the basal cAMP production (Fig. 3, top). It was noted that basal cAMP level was not affected by 2-HE, suggesting that sAC was dormant in the absence of HCO₃⁻ under the basal conditions. In contrast, forskolin-stimulated cAMP increase, reflecting TMAC activity, was attenuated by SQ22,536 (P = 0.002 vs. forskolin stimulation in the control) but was not affected by 2-HE (Fig. 3, bottom). These data together demonstrate that sAC is functional in Calu-3 cells and also validate that 2-HE could serve as a specific sAC inhibitor.

Next, we used direct functional assays of CFTR to test the notion that sAC is coupled with CFTR. We began to assess the effect of HCO₃⁻ on CFTR-mediated Cl⁻ current in whole cell voltage-clamp studies. The addition of 25 mM NaHCO₃ into the bath stimulated whole cell current mean density from 90.9 ± 18.0 to 138.8 ± 26.6 pA·pF⁻¹·100 mV⁻¹ (n = 9), and slope conductance increased 54.4 ± 9.6% compared with before the addition of HCO₃⁻ (P = 0.005) (Fig. 4). The reversal potentials of basal currents were 25.6 ± 1.6 mV, which are close to ECl (−31.9 mV) and consistent with CFTR-mediated Cl⁻ current. Given a HCO₃⁻/Cl⁻ selectivity of 0.1 (17), adding 25 mM HCO₃⁻ to the bath is predicted to shift equilibrium potential only from ECl (−31.9 mV) to −30.5 mV, a difference too small to measure. This may explain our observation that the reversal potentials of HCO₃⁻-stimulated currents (26.5 ± 1.5 mV) were not significantly different from those of basal currents. In the presence of glibenclamide, a CFTR channel blocker, 25 mM NaHCO₃ was not able to stimulate whole cell mean current density from 88.6 ± 20.7 to 98.8 ± 26.2 pA·pF⁻¹·100 mV⁻¹ (n = 3), and slope conductance increased 12.0 ± 11.1% compared with before addition of HCO₃⁻ (P = 0.19). The Cl⁻ permeability, glibenclamide sensitivity, and linear I-V relationship together suggest that NaHCO₃-stimulated current is conducted by CFTR channels. Cells treated with 2-HE had virtually no increase in current in response to 25 mM NaHCO₃ (mean current density from 91.5 ± 6.7 to 99.0 ± 13.5 pA·pF⁻¹·100 mV⁻¹, n = 3), and slope conductance increased 5.0 ± 7.5% (P = 0.28), implicating sAC being physiologically coupled to CFTR activity. An alternative explanation could be that 2-HE directly binds to and blocks the CFTR channel, because 2-HE may have other effects (8). This possibility seems less likely because adding 2-HE had no effect on forskolin-stimulated whole cell current. Forskolin (10 μM) stimulated whole cell current mean density from 71.2 ± 12.1 to 152.3 ± 42.1 pA·pF⁻¹·100 mV⁻¹ (n = 7), and slope conductance increased 106.3 ± 24.9% compared with before the addition of forskolin (P = 0.0002). In the presence of 2-HE, forskolin stimulated whole cell current mean density from 73.2 ± 17.7 to 136.3 ± 37.7 pA·pF⁻¹·100 mV⁻¹ (n = 6), slope conductance increased 98.2 ± 20.9% compared with before the addition of forskolin (P = 0.0007). The forskolin-
stimulated slope conductance increases with or without 2-HE were not significantly different (Fig. 4C).

To determine the functional coupling of sAC and CFTR more rigorously, we examined the effect of HCO3\textsuperscript{−} on CFTR single-channel activity in cell-attached membrane patches. Adding 25 mM NaHCO3 (buffered to pH 7.4 with predetermined HEPES) in the bath robustly stimulated CFTR channels in cell-attached patches (n\textsubscript{p0} increased from 0.23 ± 0.06 to 0.83 ± 0.29, n = 22; P = 0.02). The stimulation did not seem to result from osmolarity or an electrolyte effect of NaHCO3 (29), because no effect on CFTR was observed with the addition of either 25 mM NaCl (n\textsubscript{p0} changed from 0.07 ± 0.03 to 0.04 ± 0.03, n = 4; P = 0.23) or 35 mM NaNO3 (n\textsubscript{p0} changed from 0.68 ± 0.40 to 0.66 ± 0.41, n = 5; P = 0.24). HEPES (5 mM Na\textsuperscript{+}-HEPES) used to buffer the solution had no effect on CFTR either (n\textsubscript{p0} increased from 0.06 ± 0.04 to 0.055 ± 0.032, n = 3; P = 0.40). More important, the activation was attenuated by the sAC inhibitor 2-HE (n\textsubscript{p0} increased from 0.28 ± 0.06 to 0.43 ± 0.10, n = 13, P = 0.08, Fig. 5), suggesting that sAC regulates CFTR activity. Adding 2-HE had no effect on basal n\textsubscript{p0} of CFTR channels (from 0.15 ± 0.03 to 0.20 ± 0.06, n = 22; P = 0.12), consistent with no effect of 2-HE on the forskolin-stimulated whole cell current. Adding HCO3\textsuperscript{−} had no effect on the amplitude of CFTR single-channel current (Fig. 5) as expected on the basis of little predicted effect of adding 25 mM HCO3\textsuperscript{−} on the resting potential of Calu-3 cells. Increasing the concentration of NaHCO3 to 50 mM did not seem to cause any further stimulation of CFTR channel (without 2-HE, n\textsubscript{p0} changed from 0.15 ± 0.06 to 0.68 ± 0.24, n = 6, P = 0.04; with 2-HE, n\textsubscript{p0} changed from 0.16 ± 0.06 to 0.20 ± 0.05, n = 8, P = 0.26) (Fig. 5D), indicating that external 25 mM NaHCO3 elicited maximal stimulation.

DISCUSSION

In summary, our data together demonstrate that sAC is coupled to the regulation of CFTR function in Calu-3 cells. This coupling ties the level of intracellular HCO3\textsuperscript{−}/CO2 to the modulation of HCO3\textsuperscript{−}-conductive CFTR function by cAMP/PKA. With a more direct and relevant functional assay at the single-channel level and a sAC-specific inhibitor, our study has confirmed and extended the previous study in bovine corneal endothelium (27). It is not clear whether coupling of sAC and CFTR is present in other HCO3\textsuperscript{−}-transporting tissues. Conceivably, distinct biochemical properties of sAC may cause the underappreciation of its importance in HCO3\textsuperscript{−}-transporting and other biological processes because activators of G proteins and forskolin are two means primarily used to manipulate cAMP signaling.

Consistent with previous studies in various cell types (4, 26, 30), we found that T-sAC protein is the predominant isoform in Calu-3 cells (Fig. 1B). In contrast, RT-PCR studies showed an apparent dominance of the product corresponding to FL-sAC mRNA in the first round of RT-PCR using total RNA as the template (Fig. 1A, left) but failed to identify the T-sAC transcript because of overwhelming nonspecific products. Using the lower band as the template, which supposedly contained the RT-PCR product corresponding to T-sAC and contamination of the upper band (FL-sAC), the product corresponding to FL-sAC gene was still predominant in the second round of
PCR (Fig. 1A, right), suggesting a relative scarceness of the T-sAC mRNA and/or overwhelming contamination of the upper band (FL-sAC). Because the RT-PCRs of both FL-sAC and T-sAC were performed in the same tube, tube-to-tube variation and other factors affecting amplification efficiency were negated. Thus only the quantity and the heterogeneity of the FL-sAC and T-sAC RNA templates could account for the different abundance of the PCR products. The RNA templates of the FL-sAC and T-sAC are highly homologous; however, it has been reported that even a small difference in the composition of RNA templates might result in a three- to fourfold change in the efficiency of reverse transcription (10). An early study in the rat demonstrated that the abundance ratio of the FL-sAC to T-sAC in RT-PCR experiments was in good agreement with the data of RNA protection analysis (14), suggesting that the heterogeneity of the rat FL-sAC and T-sAC RNA templates does not have a significant impact on the RT-PCR amplification and that the RT-PCR data reflect the actual abundance of the mRNA of FL-sAC and T-sAC. Whether this finding is also true for the human sAC needs further confirmation.

The overlapping regulatory properties of the TMAC isoforms and the lack of isoform-specific agonists and antagonists remain a major hurdle for functionally distinguishing TMAC isoforms, particularly in studies of the human cells, in which knockout of a specific adenylyl cyclase isoform is not practicable. sAC possesses a peculiar regulatory property, i.e., the sensitivity to \( \text{HCO}_3^- \), which can be exploited to distinguish sAC from other adenylyl cyclases. However, because \( \text{HCO}_3^- \) concentration and pH in a solution are interdependent, adding \( \text{HCO}_3^- \) might result in pH alteration. In the present work, possible pH change resulting from the addition of \( \text{HCO}_3^- \) was carefully minimized. Yet, the minimal pH change could still complicate the data interpretation. To distinguish sAC from other adenylyl cyclases rigorously, we used a sAC blocker, 2-HE. 2-HE has been found to block both purified native and recombinant rat sAC (2, 23), but whether 2-HE has any effect on TMACs is not clear. In our studies, we found that 2-HE had no effect on forskolin-stimulated cAMP production, reflecting the TMAC activity; in contrast, 2-HE completely blocked \( \text{HCO}_3^- \)-stimulated cAMP production (Fig. 3). These data suggest that 2-HE could be used as a specific blocker to distinguish sAC from TMACs. Subsequently, we used 2-HE to disrupt sAC function to test whether sAC is functionally coupled with CFTR. We have demonstrated that 2-HE could attenuate the \( \text{HCO}_3^- \)-stimulated CFTR activity in both whole cell voltage-clamp and cell-attached single-channel studies (Figs. 4 and 5), consistent with the finding that sAC regulates CFTR function. We also conducted control experiments to rule out the possible effect of 2-HE on the CFTR channel itself and/or PKA. We found that 2-HE had no effect on forskolin-stimulated CFTR-mediated whole cell current, arguing against any effect of 2-HE on CFTR itself and/or PKA (Fig. 5). This finding is further supported by the observation that 2-HE had no effect on basal CFTR single-channel activity (see RESULTS). Taking all of these results together, we conclude that sAC is functionally
coupled to CFTR. Nevertheless, pharmacological reagents such as 2-HE have nonspecific effects (8). Even with carefully designed controls as described in our studies, conclusions drawn from these studies are inherently indirect and need to be substantiated further by evidence using specific molecular approaches to manipulating sAC activity, such as RNA interference.

Interestingly, HCO₃⁻ induced reasonably robust activation of CFTR while generating a rather modest cAMP increase compared with forskolin (Fig. 3). This is reminiscent of adenosine stimulation of CFTR in Calu-3 cells (11), suggesting localized cAMP signaling from sAC to CFTR. In addition, a fraction of sAC was distributed in the membrane fraction in Calu-3 cells (Fig. 2), consistent with several previous studies indicating that so-called soluble adenylyl cyclase could be membrane associated and compartmentalized in certain intracellular organelles (14, 18, 30, 31). It will be interesting to test whether sAC resides with CFTR in an apical microdomain in Calu-3 cells.

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