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Modulation of Ca^{2+}-Activated Cl^- Secretion by Basolateral K^+ Channels in Human Normal and Cystic Fibrosis Airway Epithelia

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ABSTRACT

Human airway epithelia express Ca^{2+}-activated Cl^- channels (CaCC) that are activated by extracellular nucleotides (ATP and UTP). CaCC is preserved and seems to be up-regulated in the airways of cystic fibrosis (CF) patients. In the present study, we examined the role of basolateral K^+ channels in CaCC-mediated Cl^- secretion in native nasal tissues from normal individuals and CF patients by measuring ion transport in perfused micro Ussing chambers. In the presence of amiloride, UTP-mediated peak secretory responses were increased in CF compared with normal nasal tissues. Activation of the cAMP pathway further increased CaCC-mediated secretion in CF but not in normal nasal mucosa. CaCC-dependent ion transport was inhibited by the chromanol 293B, an inhibitor of cAMP-activated hKvLQT1 K^+ channels, and by clotrimazole, an inhibitor of Ca^{2+}-activated hSK4 K^+ channels. The K^+ channel opener 1-ethyl-2-benzimidazolinone further increased CaCC-mediated Cl^- secretion in normal and CF tissues. Expression of hSK4 as well as hCACC-2 and hCACC-3 but not hCACC-1 was demonstrated by reverse transcriptase PCR on native nasal tissues. We conclude that Ca^{2+}-activated Cl^- secretion in native human airway epithelia requires activation of Ca^{2+}-dependent basolateral K^+ channels (hSK4). Co-activation of hKvLQT1 improves CaCC-mediated Cl^- secretion in native CF airway epithelia, and may have a therapeutic effect in the treatment of CF lung disease. **(*Pediatr Res* 53: 608–618, 2003)**

Abbreviations
CaCC, Ca^{2+}-activated Cl^- channel
CF, cystic fibrosis
CFTR, cystic fibrosis transmembrane conductance regulator
1-EBIO, 1-ethyl-2-benzimidazolinone
I_{sc}, equivalent short circuit current
R_{te}, transepithelial resistance
UTP, uridine 5'-triphosphate
V_{te}, transepithelial voltage

Mutations in CFTR result in defective cAMP-dependent Cl^- secretion and enhanced Na^+ absorption across CF airway epithelia, and cause progressive lung disease in CF patients. Functional expression of alternative CaCC in airway epithelia, distinct from CFTR, has been well documented in previous studies (1–8). CaCC is activated by the extracellular nucleotides (ATP and UTP) and other Ca^{2+}-increasing agonists. The fact that CaCC-mediated Cl^- secretion is preserved in CF airways makes it a potential therapeutic target to bypass the Cl^- secretory defect in CF. Enhanced CaCC activity has been detected in human and murine CF airways *in vivo*, in freshly excised CF nasal and tracheal tissues, and in primary cultures of CF airway epithelial cells (3, 9–13). Along these lines, high levels of CaCC expression in murine airways have been suggested to compensate for the lack of CFTR, thus preventing the development of lung disease in CF mice (4).

Cl^- secretion in polarized epithelia requires parallel activation of luminal Cl^- channels and basolateral K^+ channels to generate the driving force for Cl^- exit into the lumen. Two types of K^+ conductances, activated by either increase in intracellular cAMP or Ca^{2+}, have been identified in intestinal and airway epithelia (14, 15). The cAMP-dependent K^+ channel is of very small single-channel conductance (<3 pS) and is specifically inhibited by the chromanol compound 293B (15, 16). Immunohistochemistry, electrophysiological data, and

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pharmacology of the cloned KvLQT1 K^+ channel gave evidence that KvLQT1 forms the native cAMP-dependent K^+ channel in intestinal epithelia (17, 18). We have previously identified basolateral hKvLQT1 K^+ channels and their role in cAMP-dependent Cl^- secretion in human airways (19). The Ca^{2+}-regulated intermediate conductance K^+ channel has been well characterized in colonic epithelia. This K^+ channel has a single-channel conductance of ~16 pS, is inhibited by clotrimazole and charybdotoxin, and is activated by Ca^{2+} and the benzimidazolone 1-EBIO (20–23). The biophysical properties and the pharmacological profile of the cloned hSK4 K^+ channel indicated that hSK4 constitutes this native Ca^{2+}-dependent K^+ channel (23–26). However, the role of hSK4 in Cl^- secretion in the airways has not yet been investigated.

The aim of the present study was to examine the role of cAMP- and Ca^{2+}-dependent basolateral K^+ channels (hKvLQT1 and hSK4) in CaCC-mediated Cl^- secretion in native human normal and CF airway tissues. We provide evidence that both hKvLQT1 and hSK4 K^+ channels participate in CaCC-mediated Cl^- secretion in human airway epithelia, and therefore form novel targets for pharmacotherapy of the ion transport defect in CF airways.

METHODS

Ussing chamber experiments. Freshly excised nasal tissues were obtained over a period of 32 mo, from 44 normal individuals (mean age, 38.6 ± 2.5 y; range, 5–72 y; 27 males, 17 females) undergoing surgery for plastic reconstruction or sleep apnea syndrome, and from 27 CF patients (mean age, 10.9 ± 1.3 y; range, 3–38 y; 16 males, 11 females) after polypectomy. CF patients were genotyped for the following common CFTR mutations: ΔF508, R553X, N1303 K, G542X, G551D, and R347P. The following genotypes were identified: ΔF508/ΔF508 (n = 15); ΔF508/G542X (n = 1); ΔF508/G551D (n = 1); ΔF508/− (n = 2); −/− (n = 8) (− = mutation not identified). In CF patients where genetic testing did not reveal two disease-causing mutations, the diagnosis of CF was established on clinical criteria and elevated sweat Cl^- concentration. The study was approved by the Ethical Committee at the University Hospital, Albert Ludwigs University Freiburg, and nasal tissues were obtained after informed consent. Nasal tissues were kept in ice-cold buffer solution of the following composition: NaCl 127 mmol/L, KCl 5 mmol/L, D-glucose 5 mmol/L, $MgCl_2$ 1 mmol/L, Na-pyruvate 5 mmol/L, *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10 mmol/L, $CaCl_2$ 1.25 mmol/L, and albumin 10 g/L. Thin layers of nasal epithelia were dissected from the stroma and mounted into perfused micro Ussing chambers with a circular aperture of 0.95 mm^2, as described previously (27). In brief, the luminal and basolateral sides of epithelia were perfused continuously at a rate of 5–10 mL/min (chamber volume 1 mL). The bath solution had the following composition: NaCl 145 mmol/L, KH_2PO_4 0.4 mmol/L, K_2HPO_4 1.6 mmol/L, D-glucose 5 mmol/L, $MgCl_2$ 1 mmol/L, and Ca-gluconate 1.3 mmol/L. The pH was adjusted to 7.4 and all experiments were carried out at 37°C. Experiments were performed under open-circuit conditions. R_{te} was determined by applying short (1-s) current pulses ($\Delta I = 0.5\ \mu A$) and the corresponding changes in V_{te} (ΔV_{te}) and basal V_{te} were recorded continuously. Values for the V_{te} were referred to the serosal side of the epithelium. The equivalent short-circuit current (I_{sc}) was calculated according to Ohm's law from V_{te} and R_{te} ($I_{sc} = V_{te}/R_{te}$).

Experimental protocols. After mounting the tissues in Ussing chambers, an equilibration time of 30–60 min was allowed for stabilization of basal V_{te} and R_{te}. Amiloride (10 μmol/L, luminal) was added to inhibit electrogenic Na^+ absorption. Continuous bilateral bath perfusion allowed performance of consecutive measurements of nucleotide-mediated responses (UTP, 100 μmol/L, luminal) under different experimental conditions on the same tissue, *i.e.* experiments were performed in a paired fashion and each tissue served as its own control. To determine UTP-mediated peak responses, UTP was applied for 3–5 min and then washed out for ≥30 min. To test for $P2Y_2$ receptor desensitization (28), amiloride-pretreated tissues were repeatedly exposed to UTP in 30-min intervals. The initial UTP-induced I_{sc} (−20.4 ± 2.3 $\mu A/cm^2$) was not different from the UTP response after 30 min (ΔI_{sc}, −20.2 ± 4.0 $\mu A/cm^2$) or 60 min (ΔI_{sc}, −22.5 ± 3.0 $\mu A/cm^2$; n = 4), showing complete recovery of $P2Y_2$ receptors during wash out. In a subset of experiments, the UTP-mediated plateau response was determined by applying UTP continuously for 20 min. To examine the effects of cAMP-dependent co-activation on nucleotide-mediated secretion, UTP-induced Cl^- secretory responses were compared in the absence and presence of IBMX/forskolin (100/1 μmol/L, both sides). Next, we determined the effects of the basolateral K^+ channel blockers 293B (10 μmol/L, basolateral) and clotrimazole (30 μmol/L, basolateral) on UTP-activated responses in the absence and presence of cAMP activation. Finally, the effect of 1-EBIO (500 μmol/L, basolateral) on UTP-induced responses was studied in cAMP-prestimulated tissues.

Isolation of RNA and reverse transcriptase PCR. Total RNA (Micro RNA Isolation Kit, Stratagene, La Jolla, CA, U.S.A.) was isolated from native normal and CF nasal epithelia obtained by microdissection of freshly excised nasal turbinates and polyps under a stereomicroscope. Total RNA was reverse-transcribed at 37°C for 1 h using random primers and reverse transcriptase (RT) in a volume of 20 μL (Superscript RT, Invitrogen, Carlsbad, CA, U.S.A.). Sequences specific for hSK4 (405 bp, EMBL AC# AF000972) and for hCaCC-1, -2, -3 (379, 532, and 471bp, EMBL AC#: AF127036, AF127035, AF127980) were amplified by PCR (94°C for 30 s, 56–62°C for 30 s and 72°C for 60 s; 35 cycles) using the following sense (s) and antisense (as) primers: hSK4 (s), 5'-GATTTAGGGGCGCCGCTGAC-3', hSK4 (as), 5'-CTTGCCCCACATGGTGCCC-3', hCaCC-1 (s), 5'-GGGGCCATTTAAGAGTTCTG-3', hCaCC-1 (as), 5'-CTCTCCACAGTTGCCCATC-3', hCaCC-2 (s), 5'-GGGGTTATTCAGAGGTTTTG-3'; hCaCC-2 (as), 5'-CTTAGCACGGTAGAAAGGC-3'; hCaCC-3 (s), 5'-GACCCAAAGGAGCATTGCAG-3', hCaCC-3 (as), 5'-CGTGATCCGTAGCCAGCTG-3'. For comparison of CaCC-2 and CaCC-3 expression between normal and CF nasal epithelia, we performed semiquantitative multiplex RT-PCR (94°C for 2 min, 56°C for 1 min, 72°C for 1 min) with low (25 and 30) cycle numbers. CaCC-2 or CaCC-3 were co-amplified with β-actin and gel bands

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were analyzed using a densitometer (Gel Doc 2000, Bio-Rad, Hercules, CA, U.S.A.) and software (Multi-Analyst, Bio-Rad). Under these conditions, PCR reactions were not saturated. Primers for β-actin were (s) 5'-CACACTGTGCCCATCTACG-3' and (as) 5'-CTAAGTCATAGTCCGCCTAG-3' and delivered a cDNA fragment of a length of 664 bp. Intensity ratios were formed for the bands of CaCC-2/β-actin and CaCC-3/β-actin. The correct sequence of PCR products was confirmed using a Big Dye Terminator Cycle Sequencing Kit (PerkinElmer Applied Biosystem, Boston, MA, U.S.A.).

Compounds and data analysis. Amiloride, ATP, UTP, IBMX, forskolin, bumetanide, clotrimazole, and cyclopiazonic acid (CPA) were all obtained from Sigma Chemical (Deisenhofen, Germany). 1-EBIO was obtained from Biotrend Chemikalien (Köln, Germany). 293B was kindly provided by Prof. Dr. R. Greger, Physiologic Institute, Albert Ludwigs University, Freiburg, Germany. All chemicals were of the highest grade of purity available. From some individuals, transepithelial measurements were performed on more than one tissue sample. When multiple samples were studied by the same experimental protocol, data were averaged to obtain a single mean value for each individual subject. Data are shown as original recordings or as mean ± SEM (n = number of subjects). Statistical analysis was performed using paired t test. Data obtained from normal and CF tissues were compared by the unpaired t test. p Values < 0.05 were accepted to indicate statistical significance.

RESULTS

Enhanced nucleotide-mediated Cl^- secretion in CF airways. To study nucleotide-activated Cl^- secretion in native nasal tissues, the epithelia were stimulated by the 5' nucleotide UTP, which has previously been shown to increase intracellular Ca^{2+} *via* binding to $P2Y_2$ receptors expressed on the luminal membrane of airway epithelial cells (29). Table 1 shows a summary of bioelectric properties of CF and normal nasal epithelia. The allelic frequency of the most frequent CFTR mutation, ΔF508, in our CF study population was 63%, and bioelectric properties of the subgroup of ΔF508 homozygous epithelia did not differ from properties observed for the entire group of CF tissues (Table 1). As expected from previous studies (27, 30), CF nasal tissues were characterized by a significant increase in basal and amiloride-sensitive V_{te} and I_{sc}. Addition of UTP (100 μmol/L, luminal) induced a transient anion secretory response, which was characterized by an initial peak, followed by a sustained plateau. In normal tissues, UTP induced an I_{sc} peak response of -27.5 ± 3.2 μA/cm^2, which was paralleled by a significant increase in V_{te} ($\Delta V_{te} = -0.5 \pm 0.1$ mV) and decrease in R_{te} ($\Delta R_{te} = -0.4 \pm 0.1$ Ωcm^2, $n = 20$) (Fig. 1*A* and Fig. 2*C*). In contrast, addition of basolateral UTP failed to induce Cl^- secretion in native human nasal mucosa ($\Delta I_{sc} = 0.6 \pm 0.4$ μA/cm^2, $n = 5$). In CF tissues, the UTP-induced peak response was significantly increased ($\Delta I_{sc} = -41.1 \pm 6.8$ μA/cm^2; $n = 10$) compared with normal epithelia (Fig. 1*B* and Fig. 2*C*). Furthermore, UTP-activated peak responses were significantly faster in CF compared with normal epithelia (CF: 12.2 ± 1.0 s; $n = 10$ *versus* normal: 37.2 ± 2.5 s; $n = 20$; $p < 0.0001$). Increased UTP responses were also observed in the subgroup of ΔF508 homozygous epithelia ($\Delta I_{sc} = -46.3 \pm 8.5$ μA/cm^2; $n = 7$). To control for age difference, nucleotide-induced Cl^- currents were compared for a subset of tissues obtained from age-matched healthy individuals and CF patients. As observed for the entire group, nucleotide responses were significantly increased when comparing tissues obtained from the subgroup of age-matched CF ($\Delta I_{sc} = -40.6 \pm 5.8$ μA/cm^2; age: 19.2 ± 4.7 y; $n = 5$) and healthy ($\Delta I_{sc} = -22.9 \pm 3.4$ μA/cm^2; age: 22.7 ± 2.1 y; $n = 7$) individuals. The time course of nucleotide responses was examined during continuous perfusion with UTP for 20 min. In the presence of UTP, normal tissues showed a sustained Cl^- secretory response in the range of 40% of the initial peak response. In contrast, the effect of UTP was less sustained in CF tissues, where the nucleotide-induced I_{sc} returned to baseline within 10 min. (Fig. 2*A*). UTP-mediated peak responses were inhibited by bumetanide (500 μmol/L, basolateral), an inhibitor of the $Na^+/K^+/2Cl^-$ co-transporter (NKCC1), and by preincubation with the endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA, 50 μmol/L, both

Table 1. *Bioelectric properties of freshly excised nasal tissues from normal individuals and CF patients*

		Basal	Amiloride	IBMX/Fors
Normal (n = 44)	V_{te} (mV)	−0.9 ± 0.1	−0.3 ± 0.0*	−0.8 ± 0.1**
	R_{te} (Ωcm^2)	19.3 ± 1.1	21.2 ± 1.3*	20.7 ± 1.3**
	I_{sc} (μA/cm^2)	−55.6 ± 4.4	−18.3 ± 2.3*	−42.4 ± 4.3**
CF (all genotypes; n = 23)	V_{te} (mV)	−2.1 ± 0.2§	−0.3 ± 0.0*¶	−0.4 ± 0.0†
	R_{te} (Ωcm^2)	13.8 ± 1.2§	14.6 ± 1.3*	14.5 ± 1.3
	I_{sc} (μA/cm^2)	−168.4 ± 14.6§	−25.7 ± 4.2*¶	−28.8 ± 4.5†
CF (ΔF508/ΔF508; n = 13)	V_{te} (mV)	−1.8 ± 0.2§	−0.3 ± 0.1*¶	−0.3 ± 0.1†
	R_{te} (Ωcm^2)	12.8 ± 1.2§	14.5 ± 1.4*	14.3 ± 1.5
	I_{sc} (μA/cm^2)	−151.6 ± 16.6§	−17.0 ± 3.3*¶	−18.0 ± 3.5†

Basal bioelectric properties and effects of amiloride (10 μmol/L, luminal) and cAMP-dependent stimulation with IBMX and forskolin (IBMX/Fors, 100/1 μmol/L, both sides) on normal and CF nasal tissues. Values for CF tissues are given as summary of the entire CF study population (all genotypes) and for the subgroup of ΔF508 homozygous tissues (ΔF508/ΔF508).

* Statistical significance for the effect of amiloride under basal conditions (paired t test).

** Statistical significance for the effect of IBMX/Fors in amiloride pretreated tissues (paired t test).

§ Significant difference in basal properties of CF *vs.* normal nasal tissues (unpaired t test).

¶ Significant difference comparing the effect of amiloride in CF *vs.* normal nasal tissues (unpaired t test).

† Significant difference comparing the effect of IBMX/Fors in CF *vs.* normal amiloride pretreated tissues (unpaired t test).

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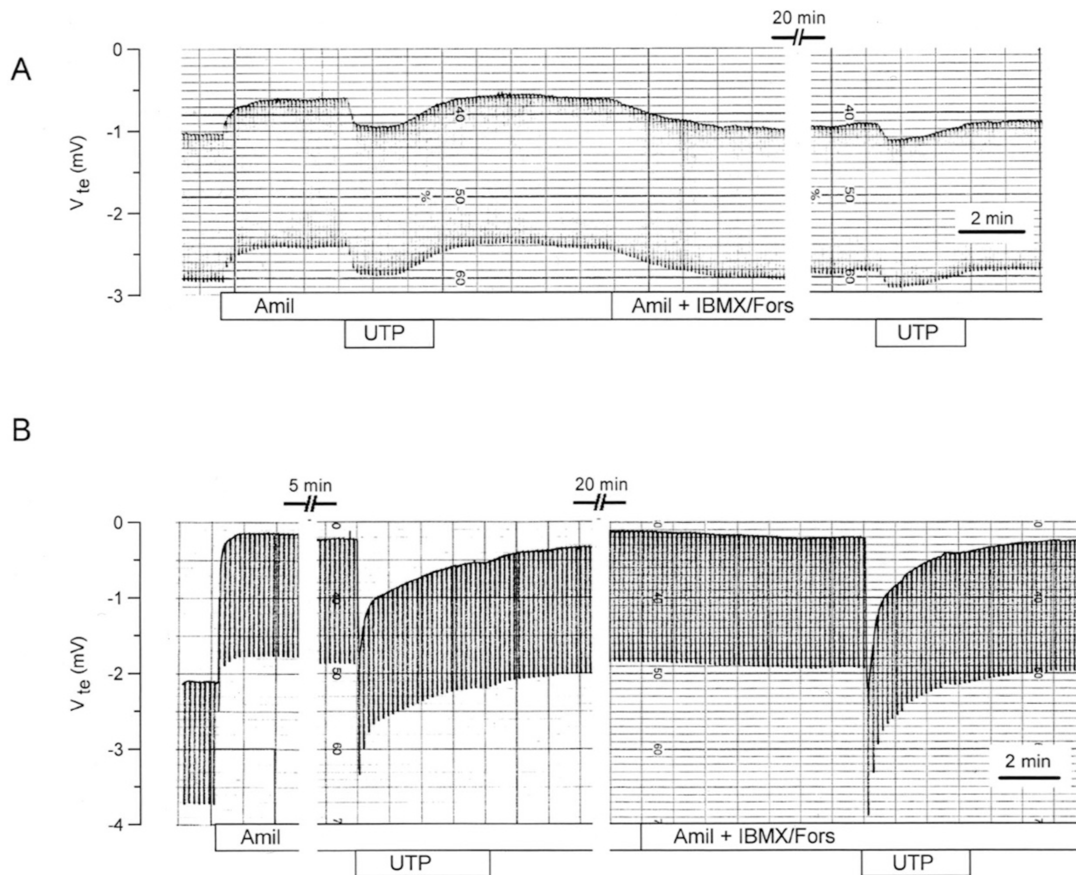

Figure 1. Original recordings of the effects of amiloride (*Amil*, 10 μmol/L, luminal), UTP (100 μmol/L, luminal), and IBMX/forskolin (100/1 μmol/L, both sides) on V_{te} and R_{te} in human (*A*) normal and (*B*) CF (ΔF508/ΔF508) nasal tissues. R_{te} was determined from the V_{te} downward deflections obtained by pulsed current injection. Time gaps between recordings were 5 min and 20 min, as indicated.

sides), showing that the UTP-induced I_{sc} was largely carried by Ca^{2+}-dependent Cl^- secretion (Fig. 2*B*). Similar observations were made when normal and CF nasal epithelia were stimulated with ATP (data not shown).

cAMP activation has opposite effects on nucleotide-activated secretion in normal and CF airway epithelia. Next, we examined the effect of cAMP activation on UTP-induced Cl^- secretion in normal and CF nasal tissues. As expected from previous studies, cAMP-dependent stimulation with IBMX and forskolin (100 μmol/L and 1 μmol/L, respectively, both sides) induced a Cl^- secretory response in normal tissues, which was inhibited by bumetanide (500 μmol/L, basolateral) (Fig. 2*D*), but failed to induce Cl^- secretion in CF tissues (Table 1; Fig. 1, *A* and *B*). After cAMP stimulation, UTP-mediated Cl^- secretion was significantly reduced in normal nasal tissues, when compared with the effect of UTP under basal conditions (Fig. 1*A* and Fig. 2*C*). In CF tissues, cAMP-dependent stimulation had an opposite effect on UTP-activated Cl^- secretion. In the presence of IBMX and forskolin, peak responses of UTP were significantly increased compared with basal conditions (Fig. 1*B* and Fig. 2*C*). Similar observations were made for the subgroup of ΔF508 homozygous epithelia where UTP-induced I_{sc} was increased to -58.5 ± 7.6 μA/cm² ($n = 7$).

Expression of CaCC isoforms in nasal epithelia. RT-PCR analysis of RNA isolated from native normal and CF nasal mucosa identified expression of the two human CaCC subtypes hCaCC-2 and hCaCC-3, whereas no transcripts could be found for the subtype hCaCC-1 (Fig. 3*A*). As a positive control for the lack of CaCC-1 expression in airway cells, we amplified CaCC-1 in cultured human HT_{29} colonic carcinoma cells (data not shown). Thus, the expression pattern for the hCaCC subtypes in the nasal mucosa is identical to that in the trachea (31, 32). Furthermore, semiquantitative (multiplex) RT-PCR was performed on RNA isolated from nasal tissues of eight healthy and six CF individuals. Either hCaCC-2 or hCaCC-3 were co-amplified together with β-actin (Fig. 3*B*). Quantitative analysis demonstrated fluorescence ratios for hCaCC-2 of 0.37 ± 0.11 (normal, $n = 8$) and 0.35 ± 0.18 (CF, $n = 6$) and for hCaCC-3 of 0.23 ± 0.10 (normal; $n = 8$) and 0.29 ± 0.14 (CF; $n = 6$). The values for normal and CF epithelia were not significantly different and thus indicate similar expression levels for hCaCC-2 and hCaCC-3 in normal and CF nasal tissues.

Role of basolateral K^+ channels in nucleotide-activated Cl^- secretion. Next, we examined the role of hKvLQT1 and the Ca^{2+}-dependent basolateral K^+ channel in regulation of UTP-activated ion transport by assessing the effects of 293B (a specific blocker for hKvLQT1) and clotrimazole (an inhibitor of the Ca^{2+}-activated K^+ channel) on UTP-induced Cl^- secretion (Fig. 4). In amiloride-pretreated normal tissues, 293B (10 μmol/L, basolateral) inhibited baseline I_{sc} slightly by 2.9 ± 0.7 μA/cm² ($n = 4$) and had no effect on UTP-mediated

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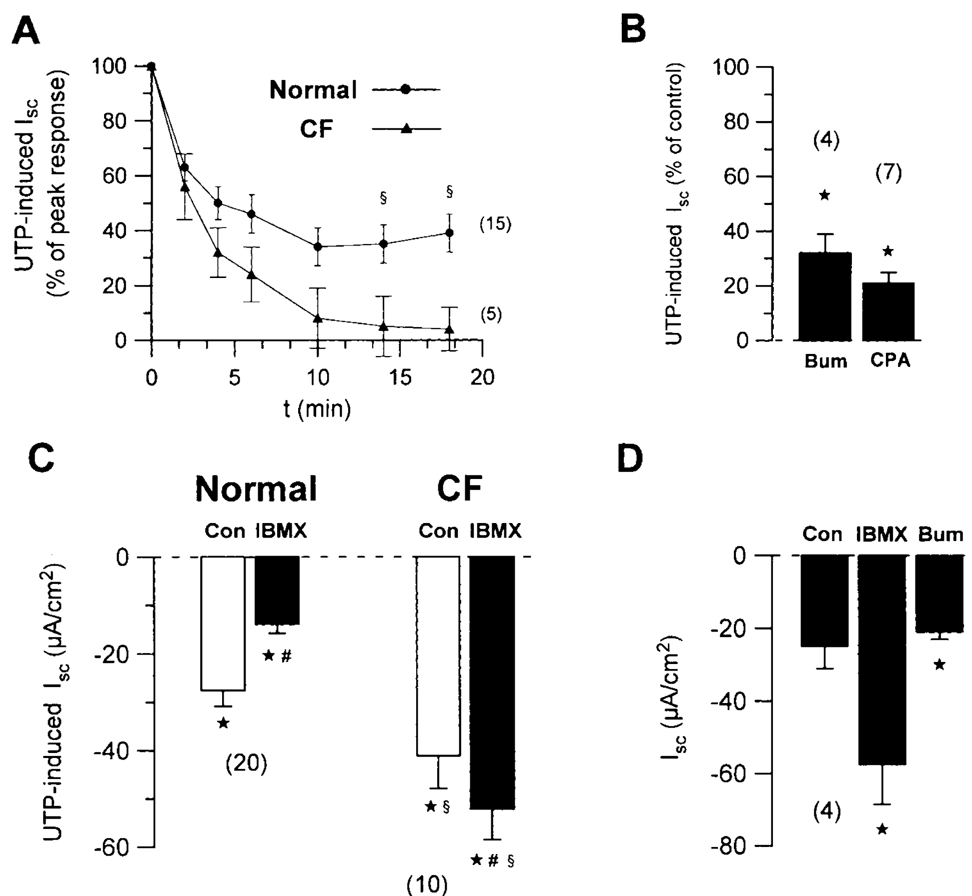

Figure 2. Summary of the effects of UTP (100 μmol/L, luminal) on normal and CF (ΔF508/ΔF508) nasal tissues after pretreatment with amiloride. (*A*) Time course of UTP-induced anion secretion (plateau response) during continuous perfusion with UTP. Data are plotted as percentage of initial UTP peak response in normal (*circles*) and CF (*triangles*) tissues. (*B*) Effects of bumetanide (*Bum*, 500 μmol/L, basolateral) and the endoplasmic reticulum (*ER*) Ca^{2+}-ATPase inhibitor cyclopiazonic acid (*CPA*, 50 μmol/L, both sides) on UTP-mediated peak responses. UTP-induced I_{sc} after pretreatment with bumetanide or CPA is shown as percentage of UTP-induced I_{sc} in the absence of the inhibitors. (*C*) Comparison of the effect of UTP-activated peak responses in the absence (*Con*) and presence of cAMP-dependent stimulation with IBMX/forskolin (*IBMX*, 100/1 μmol/L, both sides) in normal and CF tissues. UTP was washed out for ≥30 min between exposures. (*D*) Effect of IBMX/forskolin on baseline I_{sc}, and inhibition of cAMP-activated I_{sc} by bumetanide. Values are given as mean ± SE. *Significant effect of UTP, Bum, CPA, IBMX. #Significant difference comparing the effect of UTP in the absence and presence of IBMX/forskolin (paired *t* test). §Significant difference comparing the effects of UTP in normal and CF nasal tissues (unpaired *t* test).

secretory responses. In contrast, clotrimazole (30 μmol/L, basolateral) inhibited basal I_{sc} significantly by 11.3 ± 1.6 $\mu A/cm^2$ (n = 3) and abolished UTP-activated Cl^- secretion (Fig. 4, *C* and *D*), indicating that the clotrimazole-sensitive, Ca^{2+}-dependent K^+ channel, but not the 293B-sensitive, cAMP-dependent K^+ channel contributes to nucleotide-mediated secretion under basal conditions. When nasal tissues were prestimulated with IBMX and forskolin, addition of 293B completely abolished cAMP-induced I_{sc}, and reduced total I_{sc} by 55 ± 9%, as reported in our previous study (19). Moreover, UTP-induced Cl^- secretion was significantly reduced in the presence of 293B (Fig. 4, *A* and *C*). Subsequent addition of clotrimazole further reduced residual I_{sc} by 35 ± 19%, and completely abolished UTP-induced Cl^- secretion (Fig. 4, *A* and *C*). The combination of 293B and clotrimazole was equally effective in inhibiting the UTP responses as the nonspecific K^+ channel blocker Ba^{2+} (5 mmol/L, basolateral) (Fig. 4*C*). In CF tissues, prestimulated with IBMX and forskolin, addition of 293B had no effect on I_{sc}. However, the UTP-induced I_{sc} was significantly inhibited by 293B (Fig. 4, *B* and *C*). Whereas the effect of 293B on absolute UTP-activated I_{sc} was significantly increased in CF (ΔI_{sc293B} = 37.2 ± 8.6 $\mu A/cm^2$) compared with normal (ΔI_{sc293B} = 6.7 ± 2.6 $\mu A/cm^2$) airway epithelia, the fractional inhibition of UTP-mediated responses was not significantly different in normal compared with CF tissues (normal: 40 ± 12% *versus* CF: 67 ± 7%; p = 0.17). Subsequent addition of clotrimazole reduced residual I_{sc} by 46 ± 19% and inhibited UTP-activated I_{sc} completely (Fig. 4, *B* and *C*).

Next, the order of addition of 293B and clotrimazole was reversed (Fig. 4*D*). In normal tissues, prestimulated with IBMX and forskolin, clotrimazole reduced I_{sc} significantly by 55 ± 13% (n = 10). In the presence of clotrimazole, UTP-activated I_{sc} was significantly inhibited. Sequential addition of 293B further reduced nucleotide-mediated Cl^- secretion (Fig. 4*D*), and resulted in an almost complete (92 ± 5%) inhibition of I_{sc}. Similar observations were made in CF tissues, where clotrimazole inhibited I_{sc} in the presence of amiloride by 56 ± 6% (n = 7). Furthermore, clotrimazole significantly inhibited UTP-mediated Cl^- secretion (Fig. 4*D*). As observed for 293B, the inhibitory effect of clotrimazole on UTP-activated I_{sc} was

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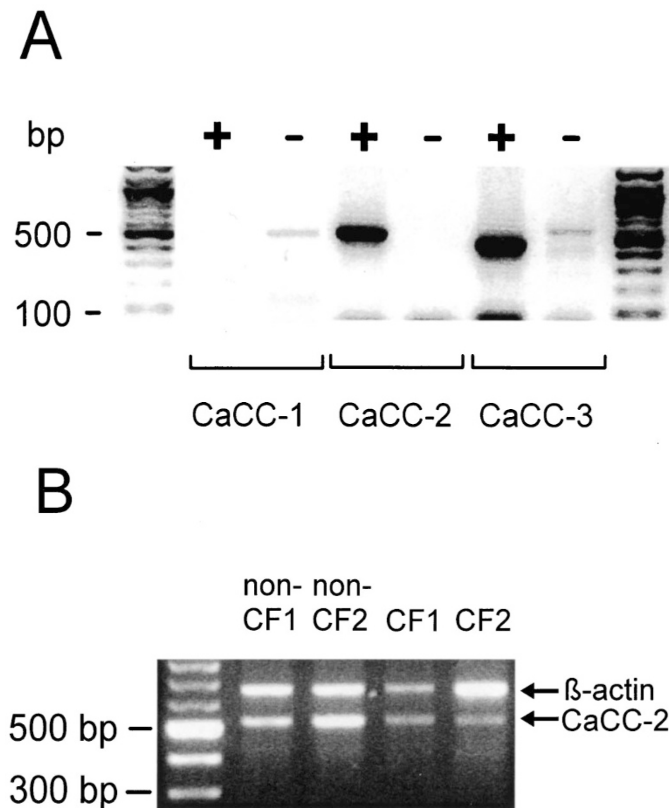

Figure 3. RT-PCR analysis of RNA obtained from freshly isolated nasal epithelia. (*A*) Analysis of the expression of human Ca^{2+}-activated Cl^- channels (hCaCC) identified transcripts for hCaCC-2 (532 bp) and hCaCC-3 (471 bp), but not for hCaCC-1. Fragments were only amplified after reverse transcription of total RNA (+), but not without reverse transcriptase (−). Subsequent sequencing of the amplified fragments confirmed the sequences of all amplified cDNA products. (*B*) Multiplex RT-PCR on RNA isolated from nasal tissues from two healthy individuals (non-CF) and two CF patients. β-actin and hCaCC-2 were co-amplified and fluorescence ratios were analyzed subsequently.

significantly increased in CF airway epithelia (CF: $\Delta I_{scClotri}$ = 34.9 ± 14.7 μA/cm^2 *versus* normal: $\Delta I_{scClotri}$ = 14.9 ± 4.1 μA/cm^2), but the fractional inhibition was similar in normal and CF tissues (normal: 70 ± 9% *versus* CF: 87 ± 4%; p = 0.18). A comparison of the effects of 293B (Fig. 4*C*) and clotrimazole (Fig. 4*D*) on nucleotide-mediated secretion in cAMP-pretreated tissues showed that clotrimazole inhibited a significantly larger fraction of the UTP-activated I_{sc} than 293B in normal and CF tissues ($p < 0.05$; unpaired t test). Subsequent addition of 293B abolished residual and UTP-activated I_{sc} almost completely, indicating that at least two different K^+ conductances are involved in UTP-activated secretion. We demonstrated by RT-PCR that the Ca^{2+}-activated and clotrimazole-sensitive K^+ channel hSK4 is expressed in native nasal epithelia (Fig. 5). Therefore hSK4 may account for the clotrimazole-sensitive, 293B-insensitive basolateral K^+ conductance in airway epithelia.

Increase of nucleotide-activated Cl^- secretion by activation of basolateral K^+ channels. The benzimidazolone compound 1-EBIO has been shown to activate Ca^{2+}-dependent (hSK4) and cAMP-dependent (KvLQT1) K^+ channels in the basolateral membrane in various expression systems aswell as colonic and airway epithelia (20, 23, 25, 26, 33, 34). We therefore examined the effect of 1-EBIO on nucleotide-induced Cl^- secretion in native nasal tissues (Fig. 6). Addition of 1-EBIO (500 μmol/L, basolateral), in the presence of amiloride and cAMP activation, induced a small but significant Cl^- secretory response in normal, but not in CF nasal epithelia (Fig. 6, *A* and *B*). However, when UTP was added in the presence of 1-EBIO, UTP-activated Cl^- secretion was significantly increased in normal and CF preparations (Fig. 6, *A* and *C*). The data demonstrate that co-activation of basolateral K^+ channels results in enhanced nucleotide-mediated Cl^- secretion *via* CaCC Cl^- channels, expressed in the luminal membrane of normal and CF airway epithelia. Therefore, co-stimulation of basolateral hSK4 and hK_VLQT1 K^+ channels may augment nucleotide-activated Cl^- secretion in CF airways.

DISCUSSION

Ca^{2+}-dependent Cl^- channels (CaCC) constitute one of the dominant ion conductances in the luminal membrane of human airway epithelia. They are expressed along with cAMP-dependent CFTR Cl^- channels and amiloride-sensitive ENaC Na^+ channels. The molecular identity of the ion channels accounting for this Ca^{2+}-dependent Cl^- conductance in airway cells has not yet been established. Previous studies identified a family of Ca^{2+}/calmodulin-activated Cl^- channels (hCaCC-1, hCaCC-2, hCaCC-3) that are predominantly expressed in the digestive and respiratory mucosa (31, 32). These previous studies and our own results indicate expression of the two subtypes hCaCC-2 and hCaCC-3 in human airway epithelia. Although the functional properties of hCaCC cDNA clones (32) do not match completely with those of endogenous CaCC in airway epithelia (35), hCaCC-2 and hCaCC-3 are the only cloned Ca^{2+}-dependent Cl^- channels that are expressed in respiratory epithelia, and are therefore candidate proteins for the Ca^{2+}-activated Cl^- conductance detected in human airway epithelium.

In airway epithelia, CaCC is activated by extracellular 5'-nucleotides (ATP and UTP) and other Ca^{2+} agonists (2, 3, 6–9, 36). Early observations demonstrated that CaCC is preserved, and CaCC activity is even increased in the airways of CF patients, making these alternative Cl^- channels pharmacological targets in CF (3, 5, 9, 12). Along these lines, it has been suggested that high levels of CaCC expression in murine airways may protect CFTR (−/−) mice from developing lung disease typically seen in CF patients (4, 10, 11). From these observations of enhanced CaCC-mediated secretion in CF airways, it was suggested that CaCC expression may be upregulated in CF (11, 37). To address this possibility, we performed semiquantitative RT-PCR for the two CaCC candidates in airway epithelia. As shown in Figure 3, both hCaCC-2 and hCaCC-3 were expressed at similar levels in normal and CF nasal epithelia. As an alternative mechanism, a recent study suggested that greater intracellular Ca^{2+} mobilization may account for enhanced CaCC activity in CF airways (13).

After inhibition of electrogenic Na^+ absorption with amiloride, freshly excised CF airway epithelia were characterized by an increased Cl^- secretory response after mucosal administra-

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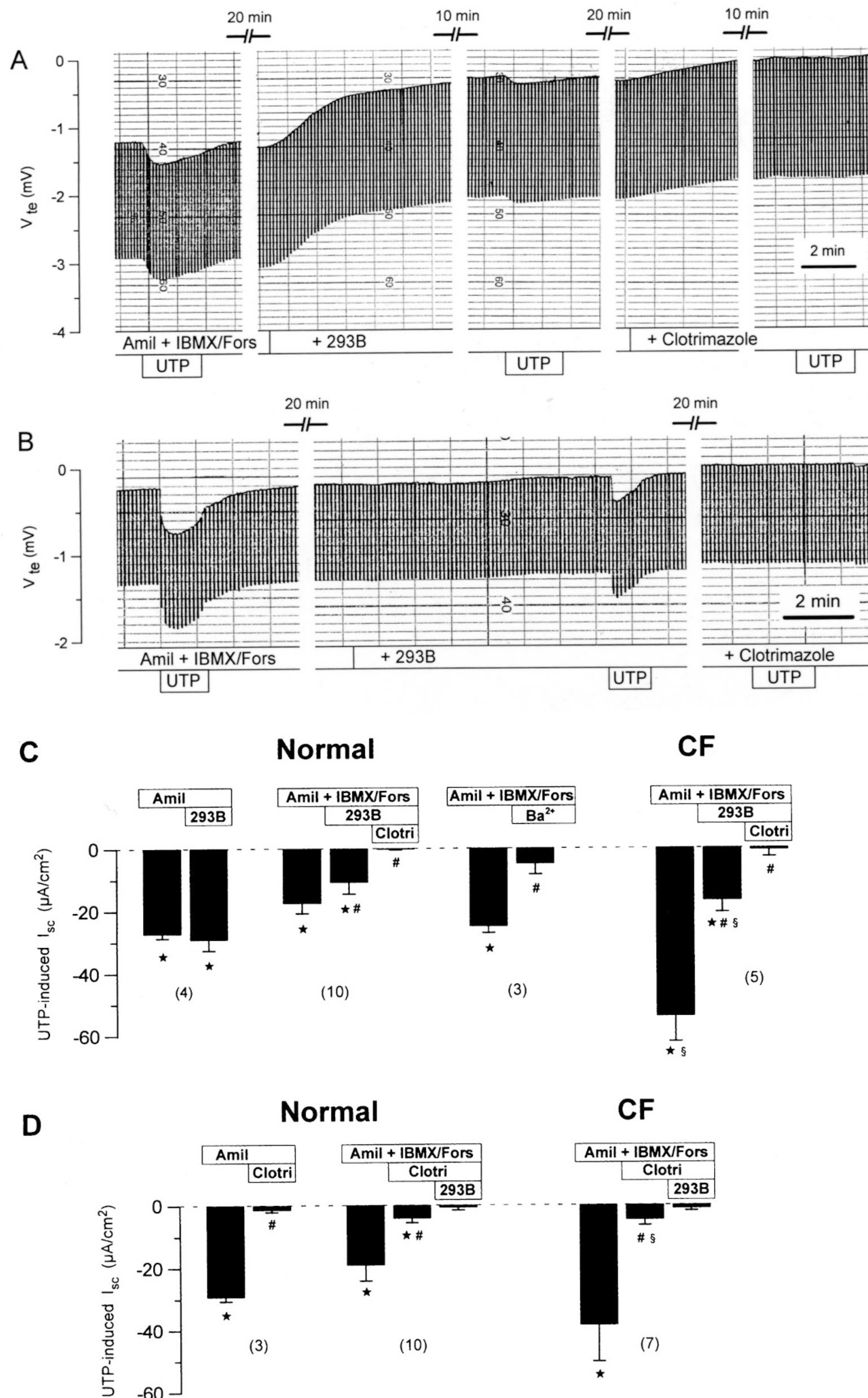

Figure 4. Original recordings and summary of the effects of the chromanol 293B (10 μmol/L, basolateral) and clotrimazole (*Clotri*, 30 μmol/L, basolateral) on UTP-activated anion secretion in normal and CF nasal tissues. All experiments were carried out in the presence of amiloride. (*A, B*) Original recordings from (*A*) normal and (*B*) CF (ΔF508/ΔF508) tissues. The effect of UTP (100 mmol/L, luminal) was examined in the presence of cAMP-dependent stimulation (IBMX/Fors, 100/1 μmol/L, both sides), and after sequential addition of 293B and clotrimazole. Time gaps between recordings were 10 min and 20 min, as indicated. (*C*) Summary of effects of 293B on UTP-induced peak responses under baseline conditions, and effects of Ba^{2+} (5 mmol/L, basolateral), and sequential addition 293B and clotrimazole on UTP-activated I_{sc} in cAMP-activated (IBMX/Fors) normal and CF tissues. (*D*) Summary of effects of clotrimazole on UTP-induced peak responses under baseline conditions, and effects of sequential addition of clotrimazole and 293B on UTP-activated I_{sc} in cAMP-activated (IBMX/Fors) normal and CF tissues. UTP was washed out for ≥30 min between exposures. Values are given as mean ± SE. *Significant effect of UTP. #Significant effect of K^+ channel blockers (293B, Clotri, Ba^{2+}) on UTP-induced I_{sc} (paired *t* test). §Significant difference comparing the effects of UTP, 293B, and clotrimazole in normal and CF nasal tissues (unpaired *t* test).

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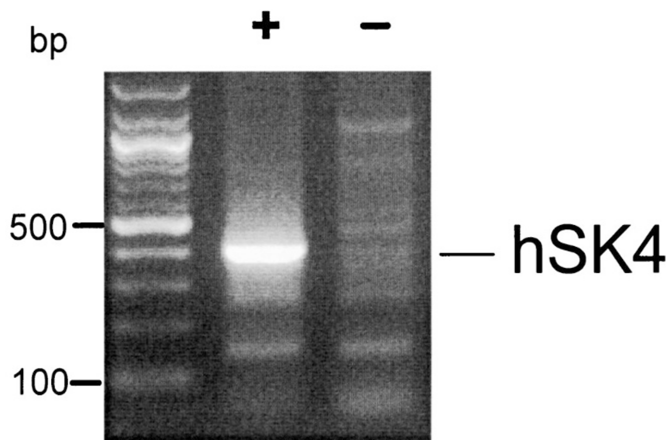

Figure 5. RT-PCR analysis on total RNA isolated from human nasal epithelia. (*A*) Analysis of expression of the cloned human Ca^{2+}-activated K^+ channel hSK4 identified transcripts of expected size (405 bp). Fragments were only amplified after reverse transcription of total RNA (+), but not without reverse transcriptase (−). The sequence of the amplified fragment was confirmed by subsequent sequencing of amplified cDNA products.

tion of ATP or UTP, confirming data from previous *in vivo* and *in vitro* studies. In contrast to earlier reports on cultured airway epithelia (3), ATP or UTP induced secretion only when applied to the luminal compartment, suggesting that $P2Y_2$ receptors are predominantly expressed on the luminal surface of freshly excised nasal mucosa. However, protein localization of $P2Y_2$ receptors in native airways by immunohistochemistry still awaits the development of specific $P2Y_2$ receptor antibodies. Both, cAMP- and nucleotide-induced lumen-negative I_{sc} was largely inhibited by bumetanide, demonstrating that both agonist pathways induced electrogenic Cl^- secretion. The onset of nucleotide-induced responses was accelerated in CF, and the peak responses were significantly greater but less sustained in CF epithelia. Activation of luminal $P2Y_2$ receptors has been shown to increase intracellular Ca^{2+} and activate protein kinase C (PKC). Previous studies have demonstrated that PKC acts as a functional regulator of CFTR-mediated Cl^- secretion in normal airway epithelial cells, and that PKC- and PKA-dependent Cl^- secretory responses are defective in CF airway epithelia (2, 13). Simultaneous measurements of intracellular Ca^{2+} and anion transport on cultured normal and CF nasal epithelial cells demonstrated that luminal $P2Y_2$ receptor agonist-mediated Cl^- secretion has at least two components: i) a transient Cl^- secretory response as a result of Ca^{2+}-dependent stimulation of CaCC, and ii) a sustained Cl^- secretory response as a result of PKC-dependent stimulation of CFTR (13). Our observation of more sustained UTP-induced Cl^- secretory responses in normal compared with CF native tissues (Fig. 2*A*) is in agreement with previous studies on cultured nasal epithelial cells (13), and suggests that the nucleotide-mediated peak response in native tissues is mediated by CaCC, whereas the plateau response reflects CFTR-dependent Cl^- secretion. Furthermore, the sustained UTP-mediated Cl^- secretory response in normal tissues was inhibited by glibenclamide (500 μmol/L, luminal; data not shown), which gives further evidence that CFTR contributes to the UTP-activated plateau response in normal airway epithelia, and suggests that the less-sustained UTP-mediated plateau response in CF tissues is due to the absence of functional CFTR in the luminal membrane.

Interestingly, cAMP-dependent activation of airway epithelia attenuated UTP-activated Cl^- secretion in normal tissues, whereas nucleotide-mediated secretory responses were further increased in CF tissues. Previous studies have shown that overexpression and stimulation of wild-type, but not ΔF508 CFTR inhibits endogenous CaCC channels in various cells (38, 39). These data suggest a regulatory interaction between CFTR and CaCC that may account for enhanced CaCC activity in CF cells. However, activation of CFTR in a native epithelium (in the presence of amiloride) is expected to result in depolarization of the luminal membrane voltage and thus reduce the electrochemical driving force for luminal Cl^- exit through an alternative (CaCC) Cl^- channels. Therefore, the resulting change in driving force could account for the attenuated CaCC-mediated Cl^- secretion observed in the presence of cAMP-dependent activation in normal epithelia. Accordingly, lack of functional and endogenously activated CFTR could explain the enhanced Ca^{2+}-activated Cl^- secretion detected in CF airways (3, 9).

Co-activation of basolateral K^+ channels along with luminal Cl^- channels is required to generate the driving force for transepithelial Cl^- secretion. Both, cAMP- and Ca^{2+}-dependent K^+ channels have been demonstrated in intestinal and airway epithelia, and their biophysical properties and pharmacological profile have been well characterized in transepithelial measurements and patch clamp studies (14, 15, 20–23, 40, 41). The cAMP-activated K^+ channel is inhibited specifically by the chromanol 293B (15, 16). Based on the pharmacology and tissue localization, the cloned hKvLQT1 K^+ channel has been suggested to constitute the native cAMP-dependent K^+ conductance in the basolateral membrane of native epithelia (17, 18). We have previously demonstrated that hKvLQT1 is expressed in normal and CF airways, and that cAMP-dependent activation of hKvLQT1 is essential for CFTR-mediated Cl^- secretion (19). Furthermore, it has been shown that the recently cloned hSK4 K^+ channel has biophysical and pharmacological properties identical to the Ca^{2+}-regulated K^+ channel in intestinal and airway epithelia. Both, hSK4 and the native channel are inhibited by clotrimazole and charybdotoxin, and activated by 1-EBIO (20–26, 42, 43). In the present study, we demonstrate that co-activation of hKvLQT1 can augment CaCC-mediated secretion in human CF airways. In the absence of cAMP activation, 293B had no effect on UTP-mediated Cl^- secretory responses. However, subsequent to cAMP stimulation, UTP-activated Cl^- secretion was significantly inhibited by the hKvLQT1 K^+ channel blocker 293B (15) in normal and CF nasal tissues. In CF tissues, due to the lack of luminal CFTR Cl^- conductance, increase of intracellular cAMP or application of 293B had no effect on I_{sc} in the absence of purinergic stimulation. However, addition of 293B resulted in significant inhibition of UTP-mediated Cl^- secretion. The data indicate that hKvLQT1 can participate in generating the driving force for CaCC-mediated Cl^- secretion in both normal and CF tissues. Accordingly, increased UTP responses subsequent to cAMP-dependent stim-

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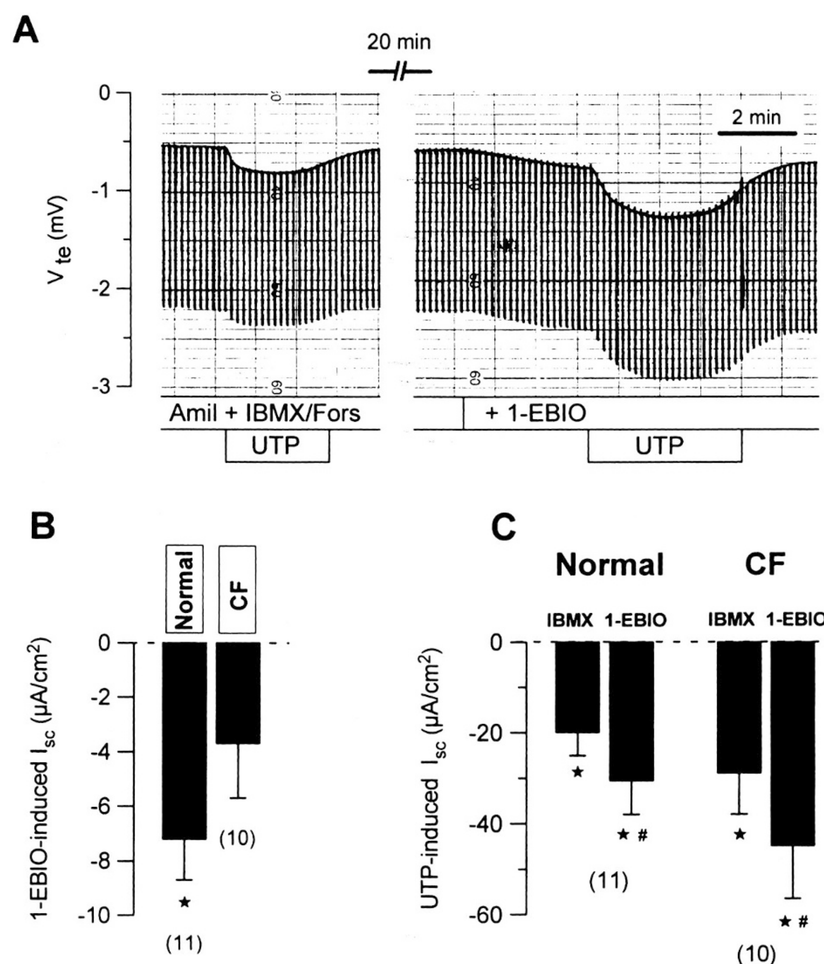

Figure 6. Original recording (*A*) of the effect of 1-EBIO (500 μmol/L, basolateral) on V_{te} and UTP-activated Cl^- secretion in cAMP prestimulated. The time gap between recordings was 20 min. (*B, C*) Summary of the effect of 1-EBIO on I_{sc} and UTP-induced I_{sc} in normal and CF nasal epithelia. All experiments were carried out in the presence of amiloride (10 μmol/L, luminal) and IBMX/forskolin (100/1 μmol/L). Values are given as mean ± SE. *Significant effect of 1-EBIO and UTP. #Significant effect of 1-EB10 on UTP-induced I_{sc} (paired *t* test).

ulation in CF tissues are likely due to co-activation of hKvLQT1.

Moreover, by RT-PCR, and by using clotrimazole as an inhibitor of hSK4 (23, 25, 26), we provide evidence that hSK4 may account for the Ca^{2+}-regulated K^+ channel expressed in the basolateral membrane of human airway epithelia, and that hSK4 plays a role in nucleotide-induced Cl^- secretion. Although 293B had no effect under baseline conditions, UTP-activated Cl^- secretion was largely abolished by clotrimazole, demonstrating that a clotrimazole-sensitive, 293B-insensitive K^+ channel is involved in CaCC-mediated secretion. Similar observations were obtained after stimulation of the tissues with IBMX and forskolin and inhibition of the cAMP-dependent K^+ channel with 293B. Under these conditions, the 293B-insensitive UTP-activated secretory response was significantly inhibited by clotrimazole. When the order of blocker administration was reversed and clotrimazole was added before 293B, clotrimazole inhibited a larger fraction of the nucleotide-mediated current compared with 293B. This finding suggests that clotrimazole may also partially inhibit the cAMP-dependent basolateral K^+ channel in native tissues, as previously shown for colonic epithelium (18, 44). Although the specific inhibitory effect of clotrimazole on hSK4 has been well documented in previous studies (21, 23, 25), the selectivity properties on other ion channels and transport proteins are largely unknown. However, the following observations indicate functional expression of at least two distinct basolateral K^+ channels, and suggest that hSK4 constitutes the Ca^+-activated K^+ channel in human airway epithelia: i) clotrimazole but not 293B significantly inhibited UTP-mediated secretion in the absence of cAMP stimulation; ii) clotrimazole inhibited the 293B-insensitive nucleotide response after cAMP activation; iii) the 293B-insensitive UTP response was inhibited by the nonselective K^+ channel blocker Ba^{2+}; and iv) hSK4 is expressed in normal and CF nasal epithelia.

Further evidence for a role of basolateral K^+ channels in CaCC-mediated airway secretion comes from experiments with the K^+ channel activator 1-EBIO, which has been shown to open hSK4 and KvLQT1 K^+ channels (23, 25, 26, 33, 34). Previous studies suggested that 1-EBIO and other benzimidazolones, like NS004, can also activate luminal CFTR Cl^- channels (20, 22, 42, 43, 45). To study the effect of 1-EBIO on basolateral K^+ channels, and exclude possible effects of 1-EBIO on I_{sc} by activation of luminal CFTR Cl^- channels,

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these experiments were performed in the presence of cAMP activation. Under these experimental conditions, CFTR Cl$^-$ channels are maximally activated, and an increase in transepithelial Cl$^-$ secretion can only be obtained by increasing the driving force for luminal Cl$^-$ exit *via* co-activation of basolateral K$^+$ channels. As recently shown for cultured human bronchial epithelial cells (41), 1-EBIO further increased V_{te} and I_{sc} in normal, but not in CF nasal tissues that were prestimulated with IBMX and forskolin. Furthermore, we demonstrate that 1-EBIO significantly increased nucleotide-mediated secretion in normal and CF airway epithelia.

Recent studies have shown that extracellular nucleotides inhibit transepithelial Na$^+$ absorption (46–48) in addition to activation of CaCC-mediated anion secretion. Here we demonstrate that apical purinergic activation by UTP co-activates basolateral Ca^{2+}-dependent K$^+$ channels (hSK4). Inhibition of Na$^+$ absorption in parallel with activation of luminal Cl$^-$ and basolateral K$^+$ channels assists the epithelium in switching from absorption under basal conditions to secretion. The finding that hKvLQT1 and hSK4 contribute to CaCC-mediated secretion identifies both K$^+$ channels as novel targets in the pharmacotherapy of CF airway disease.

In conclusion, our data suggest that both hKvLQT1 and hSK4 play an important role in generating the driving force for Cl$^-$ secretion mediated by CaCC and CFTR. Activation of wild-type CFTR attenuates CaCC responses in normal airway tissues, which is likely due to a reduced driving force for Cl$^-$ exit across the luminal membrane, and may explain the increased CaCC activity observed in CF airways. In concert with inhibitors of Na$^+$ absorption, activation of hKvLQT1 and hSK4 would promote Cl$^-$ secretion and could thus help to improve mucociliary clearance in CF airways.

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