The Amiloride-inhibitable Na^+ Conductance Is Reduced by the Cystic Fibrosis Transmembrane Conductance Regulator in Normal But Not in Cystic Fibrosis Airways

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Abstract

Cystic fibrosis (CF) airway cells, besides their well-known defect in cAMP-dependent Cl^- conductance, are characterized by an enhanced Na^+ conductance. In this study we have examined the Na^+ conductance in human respiratory tract by measuring transepithelial voltage and resistance (V_{te}, R_{te}) and by assessing membrane voltages (V_m) of freshly isolated airway epithelial cells from CF and non-CF patients. Basal amiloride inhibitable (10 μmol/liter) equivalent short circuit current ($I_{sc} = V_{te}/R_{te}$) was significantly increased in CF compared with non-CF tissues. After stimulation by forskolin (10 μmol/liter) a significant depolarization of V_m corresponding to the cAMP-dependent activation of a Cl^- conductance was observed in non-CF but not in CF airway cells. In non-CF tissue but not in CF tissue the effects of amiloride and *N*-methyl-D-glucamine on V_m were attenuated in the presence of forskolin. Also the amiloride-inhibitable I_{sc} was significantly reduced by forskolin (1 μmol/liter) and isobutylmethylxanthine (IBMX; 100 μmol/liter) only in non-CF tissue. We conclude that cystic fibrosis transmembrane conductance regulator acts as a downregulator of epithelial Na^+ channels in human airways. This downregulation of epithelial Na^+ channels is absent in CF airways, leading to hyperabsorption and to the characteristic increase in mucus viscosity. (*J. Clin. Invest.* 1998. 102: 15–21.) Key words: cystic fibrosis • epithelial Na^+ channel • cystic fibrosis transmembrane conductance regulator • airways • Cl^- conductance

Introduction

Cystic fibrosis (CF)[1] is caused by mutations in the gene of the cystic fibrosis transmembrane conductance regulator (CFTR) (1). In several previous reports CFTR was identified as a cAMP-regulated Cl^- channel (2). Above and beyond its function as a Cl^- channel, CFTR controls several other membrane conductances (3–5). Various epithelial tissues are affected in CF such as: the lungs, sweat duct, pancreas, and intestine. In these epithelia, a defect in the cAMP-activated Cl^- conductance has been reported and identified as the cause for malfunction in CF (6). However, the pathophysiology in CF lungs also includes enhanced amiloride-inhibitable Na^+ currents in parallel to impaired cAMP-dependent Cl^- conductance (7, 8). Enhanced short circuit currents in CF airways have been claimed to be due to enhanced epithelial Na^+ channel (ENaC) activity in apical membranes of respiratory epithelial cells (8). Enhanced Na^+ conductance should result in the hyperabsorption of NaCl by CF airways, provided that a Cl^- pathway different from CFTR allows for the parallel absorption of the counter ion Cl^- (8–10).

Until recently, it was unresolved how enhanced ENaC activity in CF airways is related to mutations of CFTR. Several recent reports suggest that CFTR may interfere with the ENaC (3, 11–13). ENaC expressed in MDCK cells and mouse fibroblasts was activated by cAMP (3) but it was insensitive towards cAMP when expressed in *Xenopus* oocytes (11), suggesting the requirement of additional regulatory proteins for the regulation by cAMP. Furthermore, when ENaC was coexpressed together with CFTR, the effect of cAMP on ENaC was reversed, i.e., stimulation by cAMP led to inhibition of ENaC and concomitant activation of CFTR. cAMP-dependent inhibition of ENaC was not observed for mutant forms of CFTR, such as ΔF508 or G551D (11, 14).

Although this inhibition of ENaC by CFTR has been demonstrated in several expression systems and cultured cells, nothing is known about such a channel cross-talk in airway cells. Therefore, we examined freshly isolated human respiratory epithelia by means of impalement techniques. In addition, a new modified and double-perfused micro-Ussing chamber was developed in which transepithelial membrane voltages can be measured in small biopsies of respiratory tissues. Using both techniques, respiratory tissues isolated from normal and CF nasal polyps were examined. The data presented here suggest that the Na^+ conductance in the airways of CF patients is enhanced and that this is probably due to a lack of inhibition of ENaC by mutant CFTR.

Methods

Isolation of respiratory tissue. Freshly isolated nasal epithelial cells were obtained from excised nasal polyps and nasal biopsies derived from six normal individuals and nine CF patients (ENT Clinic and Pediatric Clinic, University Freiburg). The isolation of nasal cells has been described in previous reports (8, 15). In brief, nasal tissue was washed extensively but gently in phosphate buffered solution (pH 7.4) containing (per liter) 100 mg streptomycin and 10,000 U penicillin. Parts of the tissues were used directly for impalement and Ussing chamber experiments. The remainder was used for short-term culture

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1. *Abbreviations used in this paper:* CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial Na^+ channel; I_{sc}, equivalent short circuit current; V_m, membrane voltage.

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(3–5 d) by cutting it into small pieces of ~ 3–5 mm in diameter. They were subsequently transferred into small culture dishes (35 mm). During short-term culture cells were kept at 37°C in an atmosphere of 5% CO_2/95% air. The serum-free medium was supplemented with (per liter) 10,000 U penicillin, 100 mg streptomycin, 20 μg epidermal growth factor, 15 mg endothelial growth factor, 5 mg insulin, 0.1 μmol triiodothyronine, 1 μmol aldosterone, 1 μmol hydrocortisone, and 5 mg transferrin.

Ussing chamber experiments. Freshly excised nasal polyps were kept in ice cold buffer solution with the following composition (mmol/liter): NaCl 127, KCl 5, glucose 5, $MgCl_2$ 1, Na-pyruvate 5, Hepes 10, $CaCl_2$ 1.25, albumin 10 g/liter. A piece (thin layer) of respiratory epithelium was dissected from the polyp stroma and the tissue preparation was mounted into a modified Ussing chamber. To obtain stable measurements with small pieces of tissue, we constructed a miniature chamber with a circular exposed area of 0.95 mm^2. The luminal and basolateral bath were continuously perfused at a rate of 10–20 ml/min (chamber volume 1 ml). This enabled us to sequentially examine the effect of amiloride in the absence and presence of cAMP stimulation in the same tissue. The bath solution had the following composition (mmol/liter): NaCl 145, KH_2PO_4 0.4, K_2HPO_4 1.6, D-glucose 5, $MgCl_2$ 1, Ca-gluconate 1.3. The pH was adjusted to 7.4. Bath solutions were heated by water jackets and all experiments were carried out at 37°C. To mimic the physiological situation, the Ussing chamber measurements were performed under open circuit conditions. Transepithelial voltage (V_{te}) was referenced to the serosal side. Transepithelial resistance (R_{te}) was determined by applying short (1 s) current pulses ($\Delta I = 0.5$ μA). This caused corresponding voltage deflections (ΔV_{te}). After subtraction of the ΔV_{te} obtained with the empty chamber, Ohms law was used to calculate R_{te} $[(\Delta V_{te} - V_{te}')/\Delta I]$. The equivalent short circuit current (I_{sc}) was determined from V_{te} and R_{te} by applying Ohms law again: $I_{sc} = V_{te}/R_{te}$. After stabilization of basal V_{te} and R_{te}, amiloride (10 μmol/liter) was applied to the luminal side of the nasal mucosa. The amiloride effect was entirely reversible upon wash out. Tissue preparations were then stimulated by isobutylmethylxanthine (IBMX; 100 μmol/liter) and forskolin (1 μmol/liter) and the amiloride-induced inhibition of lumen negative V_{te} was reexamined under cAMP stimulation. In four experiments with CF tissue, the amiloride effect was examined at five different concentrations (0.01, 0.1, 1, 10, and 100 μmol/liter). A concentration–response curve was constructed from the mean values and the IC_{50} was read from these curves as the concentration producing 50% inhibition of basal I_{sc}.

Microelectrodes. Freshly isolated respiratory cells were examined using microelectrodes because patch clamp analysis is not possible in these cells. Measurement of membrane voltage (V_m) by microelectrodes has been described in a previous report (8). Single-barreled borosilicate microelectrodes were pulled from filament glass (Hilgenberg, Malsfeld, Germany) using a Narishige vertical puller (Narishige, Tokyo, Japan). They had an input resistance between 80 and 200 MΩ when filled with 1 mol/liter KCl. Stable impalements could only be achieved in cells within a cluster. Impalements were only accepted when the following criteria were fulfilled: (*a*) sudden deflection of the V recording upon impalement; (*b*) concomitant increase of the input resistance by no more than 50 MΩ; (*c*) low tip potentials of < 5 mV; (*d*) stable V_m for more than 3 min; and (*e*) return of the V recording to baseline values after withdrawal of the microelectrode. V_m was measured by a high impedance (> 10^{15} Ω) electrometer (LMPR, Hampel and Rohlicek, MPI für Biophysik Frankfurt, Germany).

Compounds and analysis. Amiloride and IBMX were obtained from Sigma (Deisenhofen, Germany) and forskolin was obtained from Hoechst (Frankfurt/Main, Germany). All other chemicals were of highest grade of purity available and were obtained from Merck (Darmstadt, Germany). Data are shown as individual recordings or as mean±SEM (*n* = number of observations). Paired Student's *t* test was used for analysis of paired data in non-CF and CF tissue. To compare effects of non-CF with CF tissue unpaired *t* test was used. A *P* value of < 0.05 was accepted to indicate statistical significance.

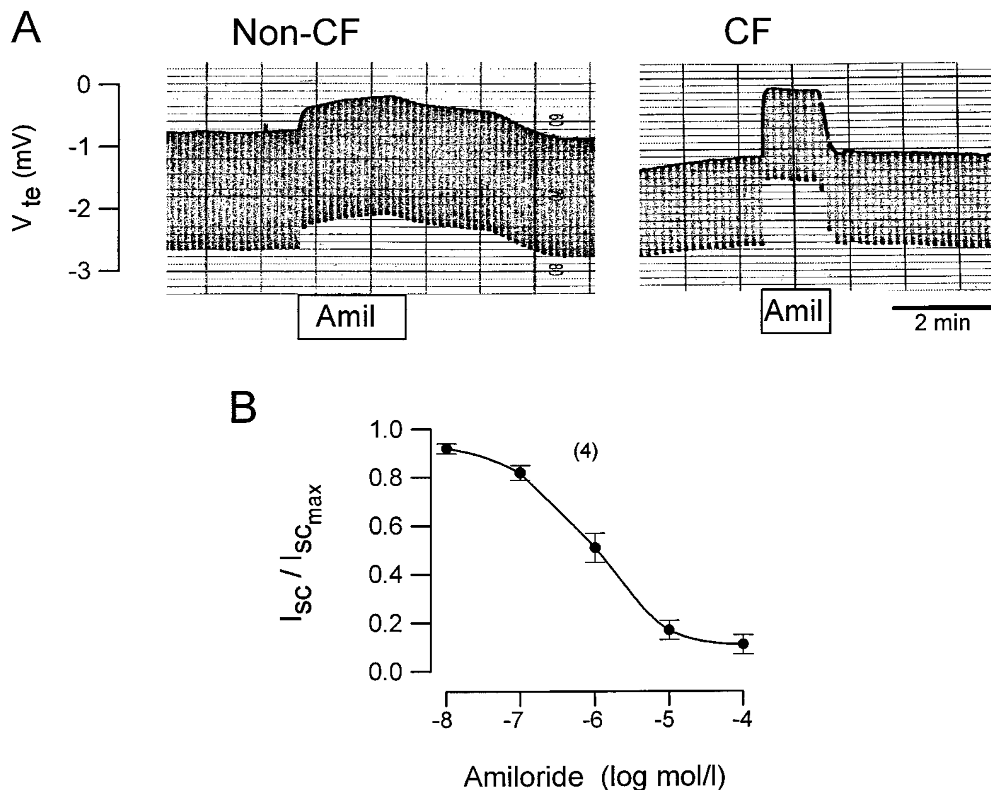

Figure 1. Amiloride-inhibitable Na^+ currents (ENaC) in non-CF and CF airway tissues. (*A*) Original recordings from Ussing chamber experiments. Amiloride reversibly reduced transepithelial potential difference (V_{te}) in non-CF and CF airway epithelia. However, basal V_{te} was enhanced in CF airways when compared with non-CF epithelia. Enhanced basal V_{te} was due to enhanced ENaC activity because inhibition of I_{sc} by amiloride was enhanced when compared with non-CF cells. (*B*) Inhibition of I_{sc} was concentration dependent with an IC_{50} of ~ 1 μmol/liter.

Results

Differences in basal Na^+ conductance in CF and non-CF airway epithelia. Under control conditions I_{sc} in non-CF airway epithelia was -24.4 ± 6.9 $\mu A/cm^2$ ($n = 8$). Basal V_{te} and R_{te} were -0.47 ± 0.2 mV and 24.7 ± 6.9 Ωcm^2, respectively. Addition of amiloride (10 μmol/liter) to the mucosal side of the epithelium reduced V_{te} in the entire series to -0.08 ± 0.03 mV and I_{sc} to -4.9 ± 2.5 $\mu A/cm^2$ ($n = 8$) (Fig. 1 *A*, see also Fig. 5 *A*). Inhibition of I_{sc} by amiloride was concentration dependent and was entirely reversible. The IC_{50} for the amiloride inhibition of I_{sc} was in the range of 1 μmol/liter ($n = 4$), reflecting the characteristic high affinity of ENaC channels towards amiloride (16) (Fig. 1 *B*). In CF tissue, basal V_{te} and I_{sc} were significantly larger: -0.9 ± 0.2 mV and -57.7 ± 12.8 $\mu A/cm^2$ ($n = 7$) (Fig. 1 *A*, see also Fig. 5 *B*). Basal R_{te} was 18.6 ± 2.5 Ωcm^2. Amiloride (10 μmol/liter) reduced V_{te} to -0.16 ± 0.03 mV and thus inhibited I_{sc} to -10.2 ± 2.5 $\mu A/cm^2$ ($n = 7$). Enhanced basal activity of ENaC channels could also be deduced from the microelectrode studies. CF cells were hyperpolarized by 10 μmol/liter amiloride more strongly than non-CF epithelial cells (Fig. 2). The data are summarized in Fig. 2 *B*. Whereas the hyperpolarization (ΔV_m) caused by the replacement of extracellular Na^+ by *N*-methyl-D-glucamine ($NMDG^+$) was only slightly larger in CF respiratory epithelial cells, the effect of amiloride was significantly larger in these cells when compared with non-CF respiratory epithelial cells. The smaller effect of $NMDG^+$ when compared with amiloride is probably due to at least two effects. First, while amiloride blocks ENaC completely, the removal of Na^+ in the extracellular medium reduces the conductance of ENaC but does not abolish this conductance. Second it is very likely that $NMDG^+$ inactivates the Na^+/H^+ exchanger which might depolarize these cells via a reduction in K^+ conductance and this will counteract amiloride induced hyperpolarization. Thus, the amiloride-inhibited Na^+ conductance probably is enhanced in CF airway epithelia under control conditions.

Effects of cAMP in non-CF and CF airway cells. Airway epithelial cells from non-CF and CF patients were stimulated by forskolin, which enhances intracellular cAMP by stimulation of the adenylate cyclase. In non-CF cells forskolin induced a slight depolarization (Fig. 3 *B*), most likely due to the activation of a depolarizing Cl^- conductance. Further evidence for the cAMP-dependent activation of CFTR Cl^- conductance in non-CF cells came from experiments in which 80% of the extracellular Cl^- was replaced by the impermeable anion gluconate (low chloride = 30Cl in Fig. 3). This caused a moderate depolarization under control conditions in CF and non-CF airway cells by some 4–10 mV. The depolarization was enhanced in forskolin-pretreated non-CF but not in CF respiratory cells. The fact that a depolarization was observed in CF airway epithelial cells under resting conditions (Fig. 3, *A* and *C*) indicates that these cells possess some Cl^- conductance which is not increased by cAMP.

Similar experiments were also performed in epithelial sheets mounted in Ussing chambers. Activation of the cAMP signaling pathway by IBMX (100 μmol/liter) and forskolin (1 μmol/liter) significantly increased I_{sc} and V_{te} in non-CF tissues from -23.0 ± 6.4 to -39.2 ± 10.4 $\mu A/cm^2$ (see Fig. 5 *A*) and -0.5 ± 0.2 to -0.8 ± 0.2 mV ($n = 8$). In CF tissues, no significant increase of I_{sc} by IBMX and forskolin was observed (see Fig. 5 *B*). These data indicate a lack of the CFTR-Cl^- conductance in CF airway epithelial cells.

Inhibition of epithelial Na^+ conductance by CFTR in non-CF but not in CF airways. Effects of amiloride on I_{sc} were examined before and after cAMP-dependent stimulation of airway epithelia (Figs. 4 and 5). In non-CF epithelia, stimulation of the cells by forskolin and IBMX significantly attenuated the

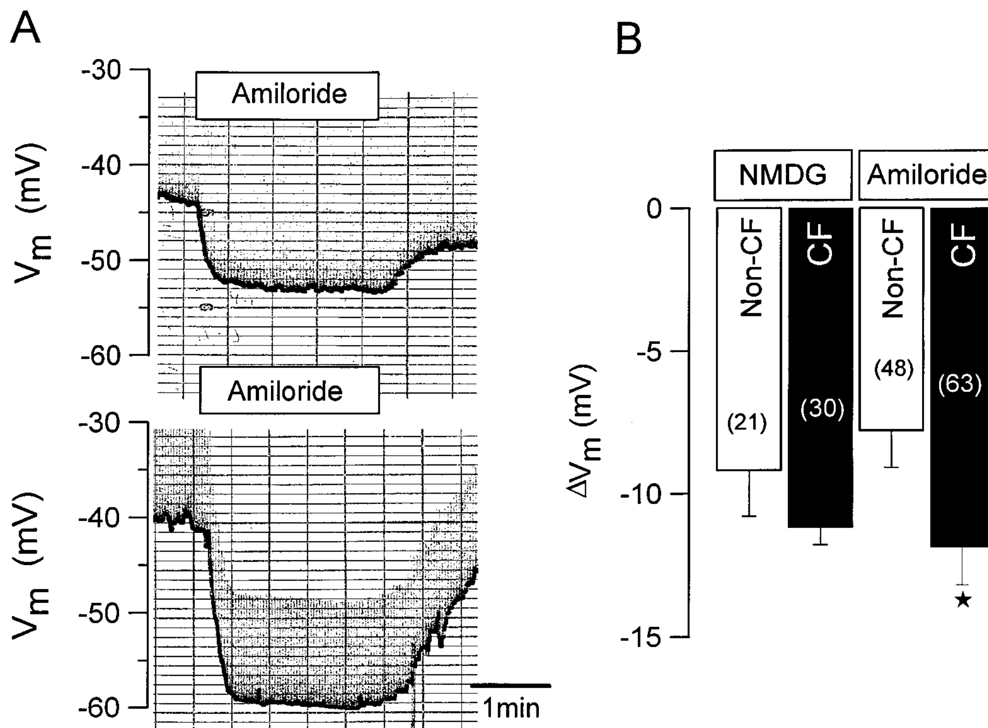

Figure 2. Hyperpolarization in respiratory cells (V_m). (*A*) Amiloride induced hyperpolarization in non-CF and CF respiratory cells. (*B*) Summary of the hyperpolarization induced by amiloride and replacement of extracellular Na^+ by *N*-methyl-D-glucamine ($NMDG^+$). *Effects of amiloride were significantly enhanced in CF airway epithelial cells. Numbers in parentheses are the number of cells recorded.

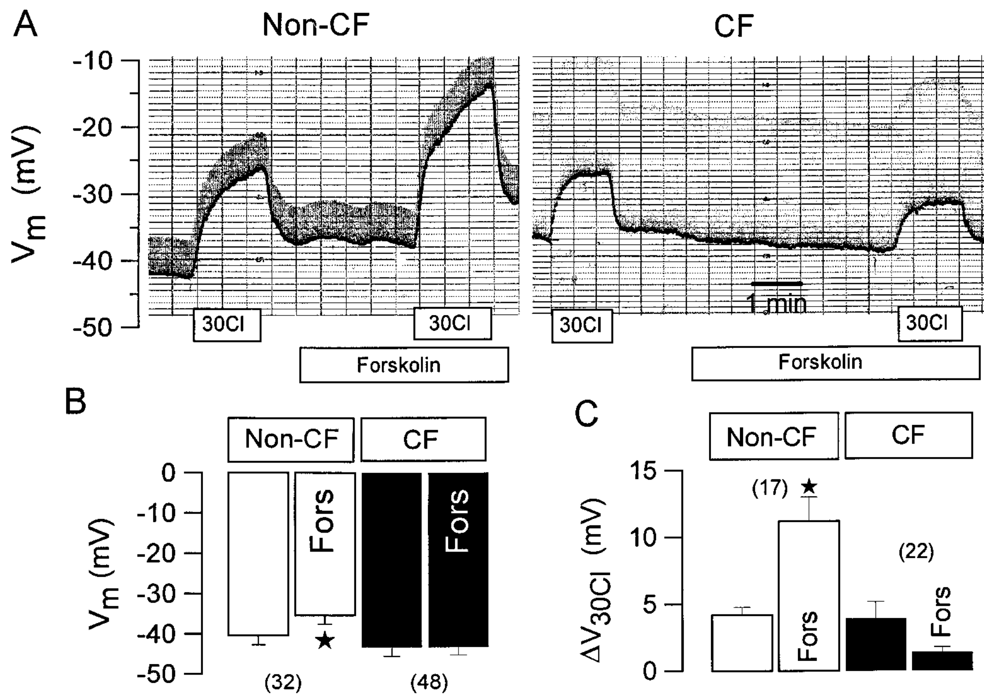

Figure 3. Effects of forskolin on non-CF and CF respiratory epithelial cells. (*A*) Depolarization of non-CF and CF epithelial cells by partial replacement of extracellular Cl^- by gluconate (*30Cl*, low chloride). Depolarization was enhanced in non-CF epithelial cells after activation with forskolin, whereas this was not observed for CF cells. (*B*) Summary of the forskolin-induced depolarization which was only observed for non-CF respiratory cells. (*C*) Summary of the depolarization due to low chloride (*30Cl*), which was significantly enhanced in non-CF epithelial cells after stimulation with forskolin.

effects of amiloride on V_{te} (Fig. 4 *A* and Fig. 5 *A*). In the presence of the secretagogues, amiloride (10 μmol/liter) reduced I_{sc} only from −38.2±10.3 to −28.2±8.5 μA/cm², whereas in the absence of any stimulant the amiloride effect was significantly larger and the inhibition was from −24.4±6.9 down to −4.9±2.5 μA/cm² (n = 8). In CF tissue, the observations were entirely different. The amiloride-inhibitable I_{sc} was significantly enhanced under basal conditions. In most preparations a further increase of I_{sc} was observed within the first 40 min of the experiment, and forskolin increased this current component even slightly further (Fig. 4 *B* and Fig. 5 *B*). In the presence of forskolin, amiloride (10 μmol/liter) reversibly reduced

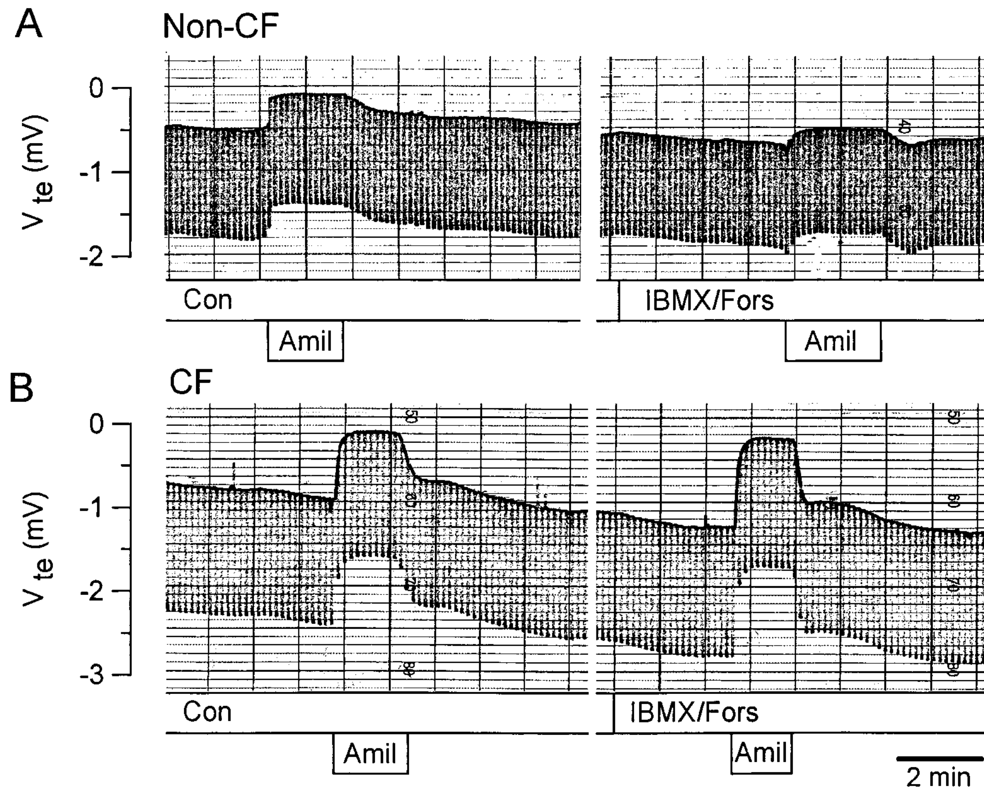

Figure 4. Attenuation of ENaC activity by CFTR in non-CF but not in CF airways. (*A*) In a non-CF respiratory tissue, stimulation with IBMX and forsolin increased transepithelial membrane voltage (V_{te}) and the effect of amiloride was attenuated after cAMP activation. (*B*) In a CF epithelium, V_{te} was also enhanced by cAMP stimulation. In contrast to non-CF tissue, the amiloride effect was augmented after addition of IBMX and forskolin.

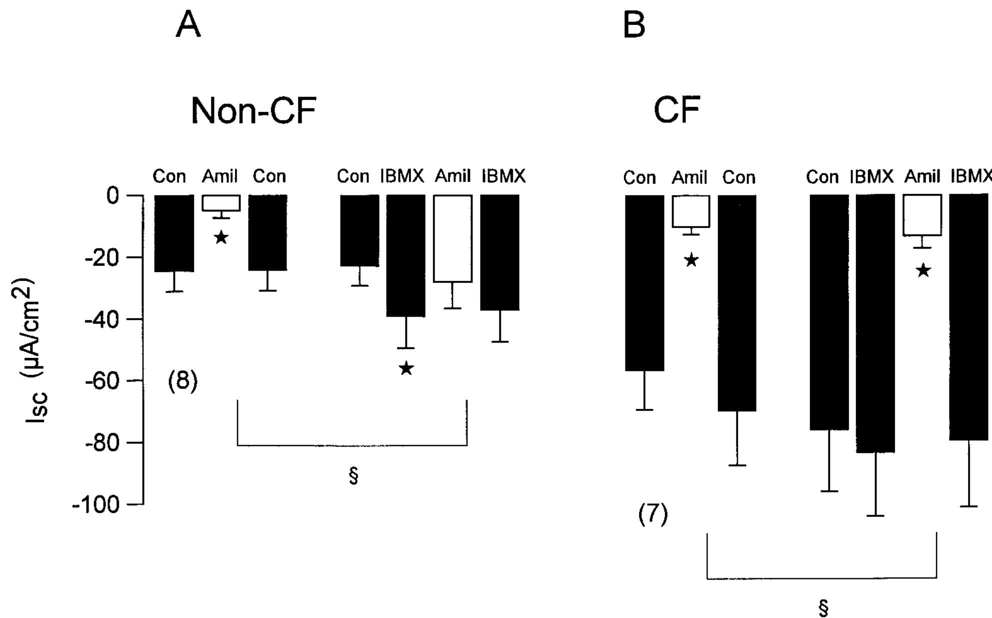

Figure 5. Attenuation of ENaC activity by CFTR in non-CF but not in CF respiratory cells. Summary of the amiloride effect on I_{sc} in the absence and presence of IBMX and forskolin, as calculated from the experiments shown in Fig. 4. (*A*) In non-CF airway tissue, amiloride significantly (*) inhibited I_{sc} under control conditions. Stimulation by IBMX and forskolin significantly increased I_{sc} (*) and the amiloride effect was significantly reduced (§) after cAMP activation. (*B*) In CF airways, basal amiloride inhibitable I_{sc} was significantly increased compared with controls (*). IBMX and forskolin increased I_{sc} slightly, but, in contrast to non-CF tissue, the amiloride effect was significantly enhanced (§) after cAMP activation.

I_{sc} from -83.3 ± 20.6 to -13.3 ± 3.9 $\mu A/cm^2$ and decreased V_{te} significantly from -1.3 ± 0.3 to -0.2 ± 0.05 mV (n = 7). Although the amiloride effect appeared significantly enhanced in stimulated CF tissues, we do not think that this reflects an increase of ENaC by stimulation, because I_{sc} became larger with time (compare with increase in control I_{sc} in Fig. 5 *B*).

Very similar observations were made in the impalement studies (Fig. 6). The amiloride-induced hyperpolarization was comparable in CF airway cells in the absence and presence of forskolin. However, it was significantly reduced in non-CF airway cells in the presence of forskolin. Replacement of Na^+ by $NMDG^+$ caused similar hyperpolarizations in CF airway cells both in the presence and absence of forskolin (Fig. 6), but hyperpolarized significantly less in non-CF cells in the presence of forskolin. Thus, the amiloride-inhibitable Na^+ conductance apparently is reduced when CFTR is activated in non-CF epithelia.

Taken together, these data indicate that CFTR-dependent downregulation of ENaC does not take place in the airways of CF patients which may explain enhanced Na^+ conductance found in this tissue.

Discussion

This study was undertaken to examine the putative interaction of CFTR and ENaC in respiratory cells of CF patients and controls. Obviously whole cell patch clamp studies and the direct measurement of ionic conductances would have been the method of choice. In our experience, however, it was close to impossible to obtain GΩ seals in these fresh cells, which all showed ciliar beating. Therefore, we examined these cells by the microelectrode technique. Stable impalements were possible and semiquantitative information on fractional conductances could be obtained by ion replacements. However, it is clear that the hyperpolarization caused by amiloride is not a direct indicator of the absolute magnitude of ENaC conductance. For this reason we supplemented our studies by Ussing chamber experiments. This technique allows the measurement of transepithelial membrane currents (I_{sc}), although, due to imperfect edge sealing, the absolute magnitudes of V_{te} and R_{te} are certainly underestimates. The results obtained by both methods match closely, indicating that the ΔV_m values observed in the impalement studies represent corresponding conductance changes.

Enhanced ENaC activity in parallel with an impaired cAMP-activated Cl^- conductance are pathophysiological hallmarks of CF in airway function. Since mutations of CFTR are the cause for CF, enhanced Na^+ conductance in CF airways should somehow be related to CFTR and its mutations as they occur in CF. Several recent reports focus on this unresolved issue and demonstrate that CFTR, when activated by cAMP, downregulates ENaC activity (3, 11–13). Although this reciprocal regulation of CFTR and ENaC has been demonstrated in various types of epithelial and nonepithelial cells, it has not been shown for respiratory epithelial cells, where it probably plays an important role in the regulation of ion transport and thus may have a major impact on the pathophysiology of CF.

These data indicate that stimulation of the airways by secretagogues leads to activation of CFTR and an increase of apical Cl^- conductance. This is probably of specific importance for the serous type of submucosal gland cells, as expression of CFTR is predominant in these cells (17, 18). These cells are probably the target of stimulation and secrete electrolytes. In contrast, surface epithelial cells expressing ENaC and very limited amounts of CFTR are thought to be responsible for absorption of electrolytes (19). Given the fact that both ENaC and CFTR are coexpressed in the same cell, Na^+ conductance will be turned off by the activation of CFTR and the cross-talk between CFTR and ENaC, with the consequence of a decrease of electrolyte absorption or maybe even a switching to NaCl secretion. Taken together, inhibition of ENaC by CFTR in the airway epithelium may reflect an important mechanism by

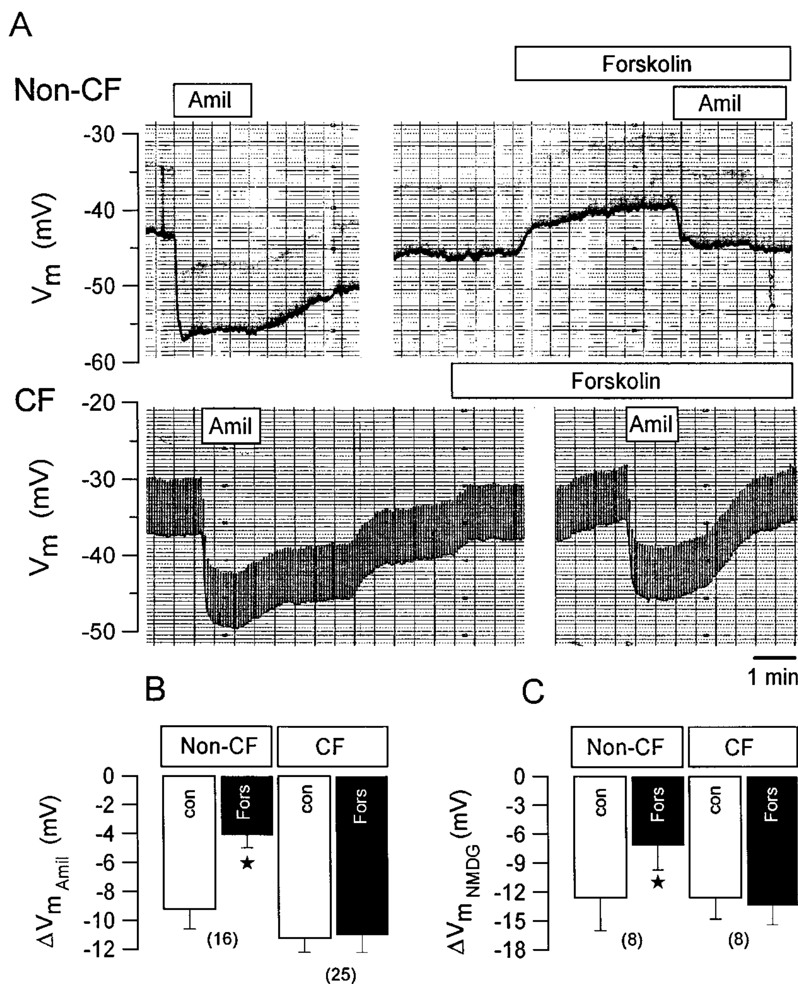

Figure 6. (*A*) Inhibition of ENaC by CFTR in respiratory epithelial cells. In a non-CF cell, amiloride hyperpolarized significantly more in the absence than in the presence of forskolin. The effects of amiloride on V_m were essentially independent of forskolin in CF airway cells. Summary of the amiloride (*B*) and $NMDG^+$ (*C*) induced hyperpolarization of V_m as measured in non-CF and CF airway epithelial cells. In the presence of forskolin, both effects of amiloride and $NMDG^+$ were significantly attenuated in non-CF but not in CF airway epithelial cells.

which the airways can switch from absorption to secretion during stimulation with secretagogues.

The short circuit current data shown in this paper indicate that CFTR-dependent downregulation of ENaC does not take place in the airway epithelium of CF patients. This may simply be explained by the fact that mutant CFTR is not able to downregulate ENaC, as already shown in other reports (11, 14). Previous studies also indicate that CFTR, when measured in native cells and under resting conditions, is already largely activated (17, 20). Therefore, in non-CF respiratory epithelial cells ENaC might be already inhibited under resting conditions while this is not the case for CF epithelial cells. This idea is in good agreement with the present and previously published data showing enhanced resting Na^+ conductance in CF airways (8, 9). The present finding that the amiloride-inhibitable Na^+ conductance in CF cells was slightly enhanced during stimulation with secretagogues is comparable to the findings of a previous report (7). It is not clear currently whether this is due to a cAMP-dependent activation of ENaC channels in CF cells, which is not observed in epithelial cells expressing both ENaC and functional CFTR. In fact, recent studies demonstrated that the response of ENaC toward protein kinase A phosphorylation was inverted in the presence of CFTR, i.e., activation was converted to inactivation (21). Alternatively, the effect of cAMP on amiloride-inhibitable short circuit currents might be more indirect and due to parallel activation of a basolateral K^+ conductance, which would hyperpolarize the basolateral membrane of airway epithelial cells and thus enhance the driving force for Na^+ uptake via the apical Na^+ conductance. A cAMP-dependent K^+ conductance has been demonstrated for several types of epithelial cells (22).

The cellular mechanism of CFTR-dependent inhibition of ENaC is currently under examination and is not yet completely understood. At present it is not clear whether this inhibition is caused by direct physical interaction of both proteins or whether additional regulatory proteins are involved (14). In fact, the cross-talk between CFTR and ENaC can be even observed under cell-free conditions in excised membrane patches or planar lipid bilayers (21, 23).

CFTR-dependent inhibition of ENaC might play an essential role in regulation of electrolyte transport in the airway epithelium. The absence of inhibition of ENaC by mutant CFTR is likely to explain enhanced Na^+ conductance present in the airways of CF patients. According to our and previous data, superficial epithelial cells from CF airways possess a residual non-CFTR Cl^- conductance under resting conditions (8). This Cl^- conductance could serve as a parallel anion pathway for absorption of NaCl and thus supports the pathophysiological concept of enhanced NaCl and water absorption in the airways of CF patients (24).

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