Cystic Fibrosis Transmembrane Conductance Regulator Inhibits Epithelial Na\textsuperscript{+} Channels Carrying Liddle’s Syndrome Mutations* 

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Epithelial Na\textsuperscript{+} channels (ENaC) are inhibited by the cystic fibrosis transmembrane conductance regulator (CFTR) upon activation by protein kinase A. It is, however, still unclear how CFTR regulates the activity of ENaC. In the present study we examined whether CFTR interacts with ENaC by interfering with the Nedd4- and ubiquitin-mediated endocytosis of ENaC. Various C-terminal mutations were introduced into the three \(\alpha\), \(\beta\), and \(\gamma\)-subunits of the rat epithelial Na\textsuperscript{+} channel, thereby eliminating PY motifs, which are important binding domains for the ubiquitin ligase Nedd4. When expressed in *Xenopus* oocytes, most of the ENaC stop (\(\alpha\)-H647X, \(\beta\)-P565X, \(\gamma\)-S608X) or point (\(\alpha\)-P671A, \(\beta\)-Y618A, \(\gamma\)-P624–626A) mutations induced enhanced Na\textsuperscript{+} currents when compared with wild type \(\alpha\), \(\beta\), \(\gamma\)-ENaC. However, ENaC currents formed by either of the mutant \(\alpha\), \(\beta\), or \(\gamma\)-subunits were inhibited during activation of CFTR by forskolin (10 \(\mu\)mol/l) and 3-isobutyl-1-methylxanthine (1 mmol/l). Antibodies to dynamin or ubiquitin enhanced \(\alpha\), \(\beta\), \(\gamma\)-ENaC whole cell Na\textsuperscript{+} conductance but did not interfere with inhibition of ENaC by CFTR. Another mutant, \(\beta\)-T392X, \(\gamma\)-T593A-ENaC, also showed enhanced Na\textsuperscript{+} currents, which were down-regulated by CFTR. Moreover, activation of ENaC by extracellular proteases and xCAP1 does not disturb CFTR-dependent inhibition of ENaC. We conclude that regulation of ENaC by CFTR is distal to other regulatory limbs and does not involve Nedd4-dependent ubiquitination.

Recent studies have indicated that epithelial Na\textsuperscript{+} channels (ENaC)\textsuperscript{1} are inhibited during activation of Cl\textsuperscript{−} secretion in human airways and the colonic epithelium (1).\textsuperscript{2} These tissues coexpress both CFTR and \(\alpha\), \(\beta\), \(\gamma\)-ENaC and demonstrate that CFTR, when activated by cAMP, inhibits epithelial Na\textsuperscript{+} channels. In fact, recent studies in cells expressing both recombinant CFTR and ENaC indicate CFTR-dependent inhibition of ENaC (3, 4). Currently, several models for regulation may be proposed: (i) we have shown recently that CFTR and \(\alpha\), \(\beta\), \(\gamma\)-ENaC whole cell Na\textsuperscript{+} conductance but did not interfere with inhibition of ENaC by CFTR. Another mutant, \(\beta\)-T392X, \(\gamma\)-T593A-ENaC, also showed enhanced Na\textsuperscript{+} currents, which were down-regulated by CFTR. Moreover, activation of ENaC by extracellular proteases and xCAP1 does not disturb CFTR-dependent inhibition of ENaC. We conclude that regulation of ENaC by CFTR is distal to other regulatory limbs and does not involve Nedd4-dependent ubiquitination.

MATERIALS AND METHODS

Liddle and \(\beta\)T592M, \(\gamma\)T93A Mutations—Mutations of the rat epithelial Na\textsuperscript{+} channel subunits were generated by polymerase chain reaction. For the C-terminal stop mutations \(\alpha\)-H647X, \(\beta\)-P565X, \(\gamma\)-S608X the following sense (s) and antisense (as) oligonucleotides (5’-3’) were used: \(\alpha\)-H647X, CGACCCACCGGCTCCGG (s), AGGACAGAAAAGGGACG (as); \(\beta\)-P565X, CCCACGGGCTGGCACC (s), CGGCGGCTCCGTCGCA (as); \(\gamma\)-S608X, TGGACACACGGTGGCC (s), AGTAAAGTGGGCGAGGTC (as). C-terminal point mutations \(\alpha\)-P671A, \(\beta\)-Y618A, \(\gamma\)-P624–626A were generated using GACAGCCCTCCTGGACTCCTGACT (s), AGTATGGACATGGGAGG-CTGTCG (as) for \(\alpha\)-P671A; GCACACTGCAATGCGTGGCCTGGAGG-GCTGTC (as) for \(\beta\)-Y618A; GTGGTCGCAACAGGCCTGGCAGATCAGATGTTTGAGGCTGTCG (as) for \(\gamma\)-P624–626A. \(\beta\)T592M, \(\gamma\)T93A was created using primers 5’-GGGACGACCTGAG-3’ (s), 5’-GTTTTGTCGATCGACGTTGAGGCTGTCG (as). C-terminal point mutations were checked for correct sequences by restriction digest and by cycle sequencing (PRISM, Perkin-Elmer). cDNAs encoding \(\alpha\), \(\beta\), \(\gamma\)-ENaC and the serine protease isolated membranes (6, 7), while other studies demonstrate inhibition of ENaC by increase of cytosolic Cl\textsuperscript{−} activities (8, 9). The results of the latter study are supported by the notion that Cl\textsuperscript{−} movement through the activated CFTR Cl\textsuperscript{−} channel is essential for the inhibition of ENaC (10). Very recently, a C-terminal CFTR domain was detected that binds to PDZ domains of proteins that anchor CFTR to the cytoskeleton (11, 12). This could provide a potential mechanism through which CFTR can affect the activity of other membrane proteins. Along this line, the cytoskeleton has been suggested for a long time to participate in the regulation of the epithelial Na\textsuperscript{+} channel (13).

Nedd4-dependent ubiquitination turned out to be an important regulatory pathway for ENaC that is also of clinical relevance in Liddle’s disease (14–16). Regulation by Nedd4 includes binding of Nedd4 to a PY motif in the C terminus of all three ENaC subunits via WW domain interaction with subsequent ubiquitination- and dynamin-mediated endocytosis of ENaC (17–19). In the present paper we addressed the question to what extent the PY motifs in the three \(\gamma\)ENaC subunits contribute to the CFTR-dependent regulation of \(\gamma\)ENaC and whether elimination of respective motifs interferes with the ability of CFTR to down-regulate \(\gamma\)ENaC. This seems to be of particular importance since Liddle’s disease patients do not suffer from pulmonary symptoms that would be caused by an enhanced Na\textsuperscript{+} conductance in the airways along with hyperabsorption of the airway surface fluid. In addition, preliminary results demonstrate inhibition of \(\alpha\), \(\beta\), \(\gamma\)-ENaC by CFTR in Madin-Darby canine kidney cells (20). In this study we coexpressed various ENaC mutants that show a gain of function. We examined the inhibitory effects of CFTR on these ENaC mutants. In addition, we analyzed whether another, recently identified regulatory pathway for ENaC, the epithelial serine protease protease CAP1 (21), interferes with CFTR-dependent inhibition of ENaC.

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1 The abbreviations used are: ENaC, epithelial Na\textsuperscript{+} channel(s); CFTR, cystic fibrosis transmembrane conductance regulator; wt, wild type; l, liter; IBMX, 3-isobutyl-1-methylxanthine.

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cRNAs for CFTR, wt, and Mutant Epithelial Na\(^+\) Channel (\(\alpha\)ENaC) Subunits and xCAP1—cDNAs encoding wild type human CFTR, the three (\(\alpha\), \(\beta\), \(\gamma\)) subunits of the rat amiloride-inhibitable Na\(^+\) channel ENaC, and the Xenopus protease xCAP1 were linearized using either NotI or KpnI, and cRNA was in vitro transcribed using T7, T3, or SP6 polymerases and a 5' cap (mCAP mRNA capping kit, Stratagene).

Preparation of Oocytes and Microinjection of cRNA—Isolation and microinjection of oocytes have been described in a previous report (10). In brief, after isolation from adult Xenopus laevis female frogs oocytes were dispersed and defolliculated by a 0.5-h treatment with collagenase (type A, Boehringer, Mannheim, Germany). Subsequently oocytes were rinsed and kept in ND96 buffer (in mmol/l): 96 NaCl, 2 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES, 2.5 sodium pyruvate, pH 7.55, supplemented with theophylline (0.5 mmol/l) and gentamycin (5 mg/l) at 18 °C. Oocytes of identical batches were injected with cRNA of either wt or mutant \(\alpha\),\(\beta\),\(\gamma\)-ENaC (each subunit 10 ng), xCAP1, and CFTR (each 20 ng), respectively, after dissolving cRNAs in about 50 nl of double-distilled water (PV830 pneumatic pico pump, WPI, Berlin, Germany). Oocytes injected with 50 nl of double-distilled water served as controls. For some experimental protocols oocytes were injected with 50 ng of either sense or antisense oligonucleotides of the serine protease xCAP1 (21).

Electrophysiological Analysis of Xenopus Oocytes—2–4 days after injection oocytes were impaled with two electrodes (Clark instruments), which had resistances of 1 M\(\Omega\) when filled with 2.7 mol/l KCl. A flowing (2.7 mol/l) KCl electrode served as bath reference. Membrane currents were measured by voltage clamping of the oocytes (OCC-1 amplifier, WPI) in intervals from −90 to +30 mV in steps of 10 mV. Current data were filtered at 400 Hz (OCC-1 amplifier). Between intervals, oocytes were voltage-clamped to their spontaneous membrane voltage for 20 s. Data were collected continuously on a computer hard disc and analyzed by using the programs chart and scope (McLab, AD-Instruments, Macintosh). Conductances were calculated for the voltage clamp range of −90 to +30 mV according to Ohm’s law. During the whole experiment the bath was continuously perfused at a rate of 5–10 ml/min.

Materials and t Test—All used compounds were of highest available grade of purity. 3-Isobutyl-1-methylxanthine (IBMX), forskolin, trypsin, and amiloride were all from Sigma (Deisenhofen, Germany). The dynamin and ubiquitin antibodies were purchased from Calbiochem (Bad Soden, Germany) and Dianova (Hamburg, Germany). Sense and antisense oligonucleotides of the protease xCAP1 were synthesized by the facility of the local University and were stabilized by phosphorothioate modification. The oligonucleotides had the following (5'-3') sequence: ATGGAGCTCTTCCACTCTTC (sense) and GAGAAGTGGAAGTGCTCCAT (antisense). Statistical analysis was performed according to Student’s t test. p values <0.05 were accepted to indicate statistical significance. All experiments were conducted at room temperature (22 °C).

RESULTS

Effects of Deletion of C-terminal PY Motifs in \(\alpha\),\(\beta\),\(\gamma\)-ENaC—PY motifs in either \(\alpha\)-, \(\beta\)-, or \(\gamma\)-subunits of \(\alpha\)ENaC were deleted by either C-terminal point (\(\alpha\)-P671A, \(\beta\)-Y618A, \(\gamma\)-(P624–626)A) or C-terminal stop mutations (\(\alpha\)-H647X, \(\beta\)-P565X, \(\gamma\)-S608X). A representative record of the whole cell current from an oocyte coexpressing \(\beta\)-P565X with wild type \(\alpha\)- and \(\gamma\)-subunits is shown in the lower trace of Fig. 1. This current is enhanced when compared with that induced by \(\alpha\),\(\beta\),\(\gamma\)-ENaC (Fig. 1, upper trace). Amiloride-sensitive whole cell conductances (\(G_{\text{ENaC-mut}}\)) were calculated for the various mutations and were compared with that of \(\alpha\),\(\beta\),\(\gamma\)-ENaC (\(G_{\text{ENaC-wt}}\)). As demonstrated in Fig. 2 \(G_{\text{ENaC}}\) was significantly enhanced for \(\beta\)-P565X, \(\gamma\)-S608X, \(\alpha\)-P671A, and \(\beta\)-Y618A, while \(\alpha\)-H647X and \(\gamma\)-(P624–626)A did not produce enhanced conductances. These results confirm those of previous studies (17, 22) and indicate the importance of the C-terminal PY motif for the regulation of ENaC.

ENaC Carrying Liddle Mutations Are Inhibited by CFTR—Individual ENaC mutants were coexpressed together with CFTR, and we examined whether these ENaC Liddle mutants are still down-regulated by CFTR. As demonstrated in Fig. 3A, the whole cell conductance that is produced by \(\alpha\),\(\beta\),\(\gamma\)-ENaC and is inhibited by 10 μmol/l amiloride (A) is attenuated after stimulation of CFTR by IBMX (1 mmol/l) and forskolin (10 μmol/l). The summary (Fig. 3B) indicates significant inhibition of \(\alpha\),\(\beta\),\(\gamma\)-ENaC by CFTR, while no effects of IBMX and forskolin could be detected in the absence of CFTR. Similar to \(\alpha\),\(\beta\),\(\gamma\)-ENaC, also \(\alpha\),\(\beta\),\(\gamma\)-S608X-xENaC whole cell conductances were down-regulated upon stimulation of CFTR with IBMX and forskolin (Fig. 3B). The results from the various ENaC mutants coexpressed with CFTR are summarized in Fig. 4. It is shown that \(G_{\text{ENaC}}\) caused by each of the C-terminal stop (A) or point (B) mutants is significantly attenuated upon stimulation of CFTR by IBMX and forskolin (black bars). We, therefore, con-
include that PY motifs do not participate in CFTR-dependent regulation of ENaC.

Antibodies for Ubiquitin and Dynamin Enhanced ENaC Conductance and Do Not Interfere with the Inhibition by CFTR—In order to further examine the role of the Nedd4/ubiquitin cascade in the regulation of ENaC by CFTR, we made use of two different antibodies, which bind to either ubiquitin and dynamin. When coinjected with ENaC, both of these antibodies enhanced G_{ENaC} when compared with amiloride-sensitive whole cell conductances measured in oocytes from the

Fig. 2. Effects of C-terminal deletions and elimination of PY motifs in α-, β-, and γ-subunits of ENaC on amiloride-sensitive Na^+ currents. cRNAs encoding either of the α-, β-, or γ-mutants were coinjected with the complementary wild type subunits. The corresponding bars indicate whole cell conductances normalized for wild type ENaC values (G_{ENaC-mut}/G_{ENaC-wt}). Results are means ± S.E. (number of experiments). * indicates significant difference from G_wt.

Fig. 3. Inhibition of αβγ-rENaC (A) and αβγS608X-rENaC (B) by CFTR. Whole cell conductances were blocked reversibly by amiloride (A, 10 μmol/l). The effects of amiloride on both wt αβγ-rENaC and αβγS608X-rENaC were attenuated after activation of a CFTR Cl^- conductance by IBMX (1 mmol/l) and forskolin (IBMX/Fors, 10 μmol/l). C, summary of the amiloride-sensitive Na^+ conductance (G_{ENaC}) in Xenopus oocytes expressing αβγ-rENaC or coexpressing αβγS608X-rENaC together with CFTR. Results are means ± S.E. (number of experiments). * indicates significant effects of amiloride. # indicates significant down-regulation of ENaC by CFTR.
same batch and injected solely with \( \alpha,\beta,\gamma \)-rENaC (Fig. 5A). In another series of experiments, we coinjected \( \alpha,\beta,\gamma \)-rENaC together with CFTR and ubiquitin or dynamin antibodies. The summary of these experiments indicates that even after interfering with both intracellular ubiquitin and dynamin, CFTR is able to inhibit ENaC (Fig. 5B).

**CFTR-dependent Inhibition of \( \beta T592M,T593A \)-ENaC**—According to previous reports, a mutation in the \( \beta \)-subunit of the human ENaC (\( \beta T594M \)) causes a loss of protein kinase C inhibition and leads to salt-sensitive hypertension in the African-American population (23). We examined whether the respective mutations in the \( \beta \) ENaC \( \beta \)-subunit interferes with CFTR-dependent inhibition. \( \beta T592m,T593A \)-rENaC, when coexpressed with equal amounts of \( \alpha \)- and \( \gamma \)-subunits, led to an amiloride sensitive Na\(^+\) conductance that was significantly higher than in oocytes from the same batch injected with wt \( \alpha,\beta,\gamma \)-ENaC (Fig. 6B). Stimulation with IBMX and forskolin had no effect on \( \alpha,\beta T592m,T593A,\gamma \)-rENaC Na\(^+\) conductance (13.7 ± 1.2 versus 13.5 ± 1.9 microsiemens; \( n = 4 \)). When coexpressed with CFTR, \( \alpha,\beta T592m,T593A,\gamma \)-rENaC conductance was significantly attenuated by stimulation with IBMX and forskolin (Fig. 6, A and C). Thus, gain of function mutations of ENaC do not limit inhibition of ENaC by CFTR.

**ENaC Conductance Activated by Extracellular Trypsin Is Inhibited by CFTR**—It was shown recently that extracellular proteases activate ENaC probably by interfering with the extracellular loop of ENaC (21). We also found augmentation of \( G_{\text{ENaC}} \) by trypsin in a dose-dependent manner (Fig. 7B). In an oocyte coexpressing both ENaC and CFTR the amiloride-sensitive Na\(^+\) conductance was enhanced after exposure to 2 \( \mu \)g/ml trypsin (Fig. 7A). Upon stimulation by IBMX (1 mmol/l) and forskolin (10 \( \mu \)mol/l) and activation of a CFTR whole cell conductance, the inhibitory effect of amiloride was largely attenuated. The summary shown in Fig. 7C indicates a significant increase of \( G_{\text{ENaC}} \) by trypsin and inhibition of \( G_{\text{ENaC}} \) by CFTR. The results demonstrate the ability of CFTR to inhibit ENaC in the presence of extracellular protease activity and suggest that

![Figure 4](https://example.com/fig4.png)

**FIG. 4.** CFTR-dependent inhibition of ENaC carrying C-terminal deletions (A, B) or point mutations (A, C) in either \( \alpha \)-, \( \beta \)-, or \( \gamma \)-subunits of rENaC. A. continuous recording of the whole cell conductances produced by either point or stop mutations of ENaC and effect of amiloride before and after activation of CFTR. Summary of \( G_{\text{ENaC}} \) produced by ENaC carrying either stop (B) or point (C) mutations before (white bars) and after (black bars) stimulation with IBMX and forskolin (IBMX/Fors). Results are means ± S.E. (number of experiments). * indicates significant effects of amiloride. † indicates significant inhibition of ENaC by CFTR.

![Figure 5](https://example.com/fig5.png)

**FIG. 5.** A, effects of dynamin and ubiquitin antibodies, respectively, on \( G_{\text{ENaC}} \). Oocytes injected with ENaC and equal amounts of water (\( \text{H}_2\text{O} \)) served as controls. * indicates significantly enhanced \( G_{\text{ENaC}} \) in coinjected oocytes compared with oocytes injected with ENaC only. B, summary of \( G_{\text{ENaC}} \) in Xenopus oocytes coinjected with CFTR and dynamin or ubiquitin antibodies. \( G_{\text{ENaC}} \) is shown before (white bars) and after (black bars) activation of CFTR by IBMX (1 mmol/l) and forskolin (IBMX/Fors, 10 \( \mu \)mol/l). Results are means ± S.E. (number of experiments). * indicates significant effects of amiloride. † indicates significant inhibition of ENaC by CFTR.
the portion of $G_{\text{ENaC}}$ that is activated by trypsin is also subjected to down-regulation by CFTR.

Inhibition of ENaC by CFTR in the Presence or Absence of the Epithelial Protease xCAP1—In order to investigate the impact of the serine protease xCAP1, we blocked expression of xCAP1 by injection of xCAP1 antisense oligonucleotides. The summary of the results is shown in Fig. 8. A and B, and demonstrate attenuation of $G_{\text{ENaC}}$ in oocytes coinjected with CFTR/ENaC/xCAP1 antisense compared with oocytes coinjected with CFTR/ENaC/xCAP1 sense. However, when CFTR was activated by IBMX and forskolin, $G_{\text{ENaC}}$ was inhibited in both sense- and antisense-injected oocytes. In a second approach we overexpressed xCAP1 together with ENaC and xCAP1 together with ENaC and CFTR. The summary of the results from these experiments is shown in Fig. 8C. The data demonstrate that $G_{\text{ENaC}}$ was approximately doubled when ENaC was coexpressed with xCAP1. However, a smaller $G_{\text{ENaC}}$ value was detected when CFTR was coexpressed with both rENaC and xCAP1. It is shown that in the presence of xCAP1, CFTR was still able to down-regulate ENaC. We conclude that larger ENaC currents that are detected in the presence of protease activity are inhibited by CFTR.

**DISCUSSION**

The amplitude of the epithelial Na$^+$ current is determined by the number of ENaC channels present in the cell membrane and by the activity of membrane resident channels (18, 19, 22). Correspondingly, dual effects of Liddle’s mutation on $P_\text{Na}$ and the number of Na$^+$ channels expressed at the cell surface have been described (19). However, endocytosis of ENaC by a Nedd4/ubiquitin/dynamin-dependent pathway is probably the predominant mechanism for regulation of epithelial Na$^+$ absorption (14). Thus, a loss of Nedd4 recognition site causes enhanced Na$^+$ absorption in kidney collecting ducts and a salt-sensitive hypertension (15–17). The well known Na$^+$ feedback (24) detected in mouse mandibular duct cells occurs via activation of $G_0$ proteins through increase of intracellular Na$^+$ and probably also via Nedd4-dependent retrieval of Na$^+$ channels from the cell membrane (25). However, in Xenopus oocytes inhibition of ENaC channels by Na$^+$ occurs without changing the number of ENaC channels (2, 19).

Because Nedd4 and ubiquitination play such a central role in ENaC regulation, we examined whether down-regulation of ENaC by CFTR occurs via this regulatory axis. According to the present results, however, this seems rather unlikely, since ENaC channels containing either $\alpha$, $\beta$, or $\gamma$-subunits with defective PY motifs were inhibited during activation of CFTR. Moreover, the data indicate strong inhibition by CFTR for most of the ENaC mutants, which may suggest that the additional portion of Na$^+$ conductance that is due to limited retrieval from the cell membrane and enhanced ENaC activity is also inhibited by CFTR. Similar holds true for $\beta$T592M,$\gamma$-ENaC and ENaC currents activated by proteases. We may therefore assume that CFTR-dependent inhibition of ENaC is superior to the regulation by the ubiquitin-dependent pathway and extracellular proteases, including xCAP1. This also applies to other patients, unlike CF patients, do not show enhanced Na$^+$ conductance in their airways and therefore do not suffer from pulmonary symptoms (20).

Nedd4/ubiquitin-dependent regulation of ENaC does not provide a mechanism through which ENaC is regulated by CFTR. The relatively fast onset and reversibility of the inhibitory effects of CFTR on ENaC (10) are in good agreement with such
Additional studies are needed to show whether the recently identified PDZ domain interaction enables functional coupling of CFTR and ENaC.

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