Inhibition of epithelial Na^+ currents by intracellular domains of the cystic fibrosis transmembrane conductance regulator

K. Kunzelmann[a,*], G.L. Kiser[b], R. Schreiber[a], J.R. Riordan[b]

[a] *Physiologisches Institut, Hermann-Herder-Straße 7, 79104 Freiburg, Germany*
[b] *S.C. Johnson Medical Research Center, Mayo Clinic Scottsdale, East Shea Blvd., Scottsdale, AZ 85259, USA*

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Abstract Cystic fibrosis is characterized by an impaired cyclic adenosine 3,5-monophosphate (cAMP) activated Cl^- conductance in parallel with an enhanced amiloride sensitive Na^+ conductance (ENaC) of the respiratory epithelium. Very recently, acute downregulation of ENaC by the cystic fibrosis transmembrane conductance regulator (CFTR) was demonstrated in several studies. The mechanism, however, by which CFTR exerts its inhibitory effect on ENaC remains obscure. We demonstrate that cytosolic domains of human CFTR are sufficient to induce inhibition of rat epithelial Na^+ currents (rENaC) when coexpressed in *Xenopus* oocytes and stimulated with 3-isobutyl-1-methylxanthine (IBMX). Moreover, mutations of CFTR, which occur in cystic fibrosis, abolish CFTR-dependent downregulation of rENaC. Yeast two hybrid analysis of CFTR domains and rENaC subunits suggest direct interaction between the proteins. Enhanced Na^+ transport as found in the airways of cystic fibrosis patients is probably due to a lack of CFTR dependent downregulation of ENaC.

Key words: Cystic fibrosis transmembrane conductance regulator; Na^+ conductance; Two hybrid analysis; *Xenopus* oocytes; Cystic fibrosis

1. Introduction

Defective regulation of ion transport is a hallmark of the pathophysiology of cystic fibrosis (CF) and is caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) [11,12]. Apart from the well described defect in cAMP dependent activation of Cl^- channels, enhanced Na^+ reabsorption was found in the airways of CF patients, causing dehydration of the airways and subsequent complications like bacterial infection [2]. More recent studies have demonstrated that both CFTR and ENaC are present in the same cells of the respiratory surface epithelium and that amiloride sensitive Na^+ currents are enhanced in CF tissues [8].

There is a functional coupling between CFTR dependent Cl^- currents and Na^+ currents in ENaC expressing epithelial cells; activation of CFTR-Cl^- currents by increase of intracellular cAMP leads to inhibition of ENaC currents [7,10,13]. In the present study we further examined the possible underlying mechanisms of this CFTR dependent downregulation of ENaC currents. The results of coexpression studies in *Xenopus* oocytes suggest that intracellular moieties of CFTR are important for inhibition of Na^+ currents, probably by interacting directly with the α-subunit of ENaC.

*Corresponding author. Fax: (49) (761) 203-5191.
E-mail: kkunzel@sibm2.ruf.uni-freiburg.de

2. Materials and methods

2.1. Cloning of cDNAs

The three (α, β, γ) subunits of the rat amiloride sensitive Na^+ channel (rENaC, kindly provided by Prof. Dr. B. Rossier, Pharmacological Institute of Lausanne, Switzerland) were subcloned into pBluescript, linearized with *Not*I and in vitro transcribed using T7 promotor and polymerase (mCAP-RNA, Stratagene). PCR products from wild type CFTR or CFTR containing the G551D mutation and comprising amino acids 351–830 were blunt-end ligated (*Eco*RV) into pBluescript SK^+. Subsequently, cRNA was transcribed in vitro (cf. above) starting with methionine 394 and coinjected into oocytes together with cRNA from αβγ-rENaC.

2.2. Voltage clamp measurements in oocytes from Xenopus laevis

Oocytes were obtained from adult *Xenopus laevis*, defolliculated by 1 h treatment with collagenase (type A, Boehringer Mannheim) and subsequently injected with 10–30 ng cRNA for each subunit. Recordings were taken 2–3 days after injection. During two electrode voltage clamp recordings, I/V curves were obtained every 30 s by voltage clamping the oocyte from −90 to +30 mV in steps of 10 mV and conductances were calculated accordingly. Oocytes were continuously perfused with amphibian Ringer containing 96 mM NaCl, 2 mM KCl, 1.8 mM Ca^{2+}, 5 mM HEPES, 1 mM Mg^{2+} at pH 7.55. In some experiments, 96 mM NaCl was replaced by equimolar concentrations of *N*-methyl-D-glucamine. Statistical analysis was performed according to Student's *t*-test. P values < 0.05 were accepted to indicate statistical significance.

2.3. Cloning of cDNAs for CFTR and αβγ-rENaC for two hybrid analysis in yeast

α- and γ-subunits of rENaC were subcloned directly from pSport into pGAD424 using *Sal*I/*Bgl*II(pGAD424)-*Sal*I/*Bam*HI(α) and *Sal*I-*Bgl*II(pGAD424)-*Sal*I/*Bam*HI (γ). The α-subunit was also subcloned into pGBT9 using *Sal*I/*Pst*I. A 2 kb fragment of the β-subunit, comprising amino acids 1–638, was PCR amplified (12 cycles, Pfu-Polymerase (Stratagene), 94°C/1 min, 54°C/1 min, 72°C/1.30), blunt end ligated into pBluescript (*Eco*RV), and subcloned as a fusion protein into pGAD424 using *Bam*HI-*Sal*I(pGAD424)-*Bgl*II/*Xho*I(β) sites. Similarly, N (α-NT, amino acids 1–110) and C (α-CT, amino acids 612–700) termini of α-rENaC were generated by PCR and subcloned as fusion proteins into pGAD424 using *Bam*HI-*Pst*I (pGAD424) and *Bgl*II/*Pst*I (αNT, αCT) sites. Correct sequences were verified by sequencing. All CFTR domains were PCR amplified from pBQ (human CFTR nt 72–4721 in pBluescript [12]), blunt end ligated into pBluescript (*Eco*RV) and subsequently subcloned in-frame into both pGAD424 or pGBT9. The correct frame and sequence of the insertions into the fusion plasmids were verified by sequencing.

2.4. Two hybrid analysis in yeast

All cytoplasmic domains of CFTR listed in Table 1 were tested for direct interaction with α, β, or γ-rENaC subunits and N (α-NT) as well as C (α-CT) termini of α-rENaC in semi-quantitative yeast two hybrid assays. Interaction was assessed by the expression of reporter gene products β-gal, producing a blue colored product in the presence of X-gal, and His3p, allowing growth on media lacking histidine. The various fragments were tested by semi-quantitative yeast two hybrid interaction assays, based on the degree of induction of reporter genes β-gal and His3p. The pGBT9 plasmid contains the DNA binding domain of *GAL4* and the pGAD424 contains the activation domain of *GAL4* (Matchmaker Two-Hybrid System; Clontech, Palo Alto, CA). Plasmids were propagated in the bacterial strain XLI-blue (Stra-

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tagene, La Jolla, CA) and introduced into yeast cells (either HF7c or SFY526 strain; Clontech, Palo Alto, CA) by lithium acetate transformation. Yeast media were standard. Transformants were tested for their β-galactosidase (β-gal) activity by colony lift assay in the presence of the chromogenic substrate 5-bromo-4-chloro-indoyl-β-D-galactopyranoside (X-gal) per Clontech Matchmaker protocol. The *HIS3* reporter gene activity was assessed by streaking transformants onto solid minimal media lacking histidine; those colonies that grew without histidine expressed the *HIS3* gene. Similar results were obtained for HF7c and SFY526 strains. Several controls were included: interaction of p53-protein and large T antigen served as a positive control while lamin C cotransformed with large T antigen acted as a negative control. Moreover, single transformants were negative. β-Gal activity was determined from the time taken for colonies to turn blue in X-gal filter assays [1] at room temperature: +++, <2 h; ++, 2–5 h; +, >5 h; −, no significant β-Gal activity. All tests were performed at least three times for one combination. His3p activity was measured by percentage of colonies growing on histidine lacking medium: +++, $>60\%$; ++, 30–60%; +, 10–30%; −, no significant growth. All tests were performed at least in triplicate (n.t. indicates not tested).

3. Results and discussion

The three subunits of the rat epithelial Na^+ channel (α, β, γ-rENaC) do not contain consensus sites for phosphorylation by protein kinase A (PKA). Accordingly α, β, γ-rENaC, when expressed in oocytes of *Xenopus laevis*, were not sensitive to changes in intracellular cAMP as demonstrated in Fig. 1: replacement of extracellular Na^+ by the impermeable cation *N*-methyl-D-glucamine ($NMDG^+$) inhibited inward Na^+ conductances and hyperpolarized membrane voltages in rENaC expressing oocytes independently of stimulation with the phosphodiesterase inhibitor IBMX, thus confirming results from a previous report [10]. Similarly, current inhibition and hyperpolarization by amiloride (5 μM), a specific inhibitor of epithelial Na^+ channels, was not influenced by IBMX [10]. In contrast and as demonstrated in a previous study [10], rENaC currents were significantly reduced when coexpressed in oocytes together with wild type CFTR stimulated with IBMX. Both the effects of amiloride and $NMDG^+$ on membrane voltage and whole cell conductance were significantly reduced after activation of CFTR (Fig. 2a,c).

Disease causing mutations of CFTR, as they occur in cystic fibrosis, are able to eliminate inhibitory effects of stimulated CFTR on rENaC. This was now demonstrated also for the CFTR mutation G551D that was coexpressed with rENaC (Fig. 2b,d). Application of IBMX had no impact on amiloride sensitive Na^+ conductance, neither were amiloride or $NMDG^+$ induced current inhibition and hyperpolarization of membrane voltages changed during stimulation. Unlike another CFTR mutation (ΔF508), maturation of G551D-CFTR is not impaired [3,6,14]; thus the possible lack of G551D-CFTR expression in the oocyte membrane is unlikely to account for the lack of downregulation of rENaC by G551D-CFTR. In addition, a small but significant whole cell Cl^- conductance of 2.2 ± 0.3 μS ($n = 13$) was activated by IBMX (1 mM) in G551D-CFTR injected oocytes. Moreover, G551D-CFTR expression in oocytes could be demonstrated by immunofluorescence and immunoprecipitation (data not shown). Since the G551D mutation is located in the first nucleotide binding fold (NBF1), the results suggest that intracellular domains of CFTR located between transmembrane sequences 6 and 7 may be involved in CFTR-dependent downregulation of ENaC. To further examine this hypothesis a CFTR fragment, comprising the first nucleotide binding fold and R-domain (P1-R, amino acids 394–830) was coexpressed with rENaC in *Xenopus* oocytes. In fact, coexpression of this fragment and stimulation with IBMX significantly, albeit less than wild type, reduced ENaC currents (Fig. 3a,c). This was also demonstrated by inhibition of amiloride sensitive Na+ currents after IBMX stimulation. In addition, effects of $NMDG^+$ were significantly reduced. Notably, rENaC currents were not downregulated by IBMX stimulation when the P1-R fragment, containing the G551D mutation (G551D/P1-R) was coexpressed (Fig. 3b,d). These experiments suggest that only parts of CFTR are required for ENaC inhibition. Moreover, the present data and those

Table 1
Summary of the two hybrid analysis

CFTR domain	Amino acids	β-Gal					His				
		α	α-NT	α-CT	β	γ	α	α-NT	α-CT	β	γ
N-terminus	1–80	−	−	−	−	−	−	−	−	−	−
Cytosolic loop 1	139–194	−	−	−	−	−	−	−	−	−	−
Cytosolic loop 2	242–307	−	−	−	−	−	−	−	−	−	−
PNBF1	351–404	−	n.t.	n.t.	−	−	−	n.t.	n.t.	−	−
NBF1	404–589	−	−	−	−	−	−	−	−	−	−
R	590–830	−	−	−	−	−	−	−	−	−	−
PNBF1+NBF1+R (wt)	351–830	++	−	++	−	−	+++	−	++	−	−
PNBF1+NBF1+R (G551D)	351–830	−	−	−	−	−	−	−	−	−	−
Cytosolic loop 3	933–990	−	−	−	−	−	−	−	−	−	−
Cytosolic loop 4	1035–1102	−	−	−	−	−	−	−	−	−	−
C-terminus	1415–1480	−	n.t	n.t.	−	−	−	n.t	n.t.	−	−
PNBF2+NBF2+C-terminus	1151–1480	−	−	−	−	−	−	−	−	−	−

Cytoplasmic domains of CFTR listed here were tested for direct interaction with α, β, or γ-rENaC subunits and N (α-NT) as well as C (α-CT) termini of α-rENaC in semi-quantitative yeast two hybrid assays. Abbreviations: PNBF1: sequence between putative transmembrane spanning domain 6 and NBF1; NBF1: first nucleotide binding domain; R: R-domain; PNBF1+NBF1+R correspond to P1-R fragments used in *Xenopus* expression studies; PNBF2: sequence between putative transmembrane spanning domain 12 and NBF2; NBF2: second nucleotide binding domain. Interaction was assessed by the expression of reporter gene products β-Gal, producing a blue colored product in the presence of X-gal, and His3p, allowing growth on media lacking histidine. The various fragments were tested by semi-quantitative yeast two hybrid interaction assays, based on the degree of induction of reporter genes β-Gal and His3p. β-Gal activity was determined from the time taken for colonies to turn blue in X-gal filter assays [1] at room temperature: +++, <2 h; ++, 2–5 h; +, >5 h; −, no significant β-Gal activity. His3p activity was measured by percentage of colonies growing on histidine lacking medium: +++, $>60\%$; ++, 30–60%; +, 10–30%; −, no significant growth.

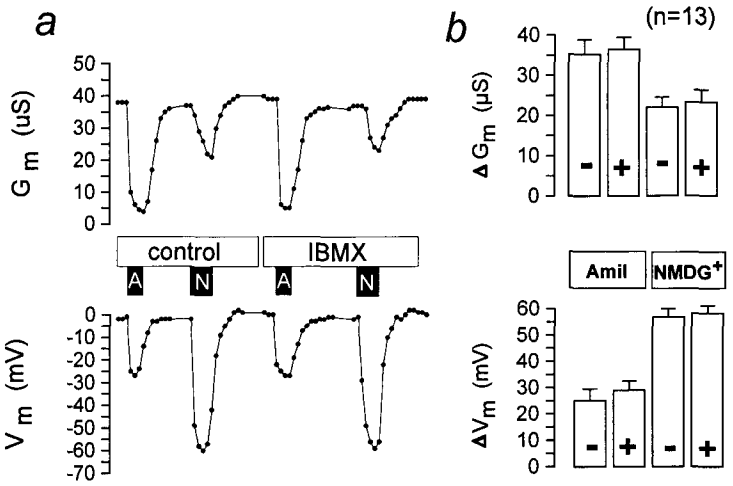

Fig. 1. Expression of epithelial Na^+ channels (ENaC) in *Xenopus* oocytes. a: Typical conductance (G_m, upper part) and voltage (V_m, lower part) recordings before (control) and after stimulation of the oocyte with IBMX (1 mmol/l). Application of 5 μmol/l amiloride (A) or replacement of extracellular Na^+ by *N*-methyl-D-glucamine (N) inhibited G_m and hyperpolarized V_m, respectively, independent of stimulation with IBMX. b: Summary of the inhibition of G_m (ΔG_m) and hyperpolarization of V_m (ΔV_m) induced by amiloride (Amil) and *N*-methyl-D-glucamine ($NMDG^+$) (paired *t*-test, $P < 0.05$). The effects were similar in the absence (−) or presence (+) of IBMX.

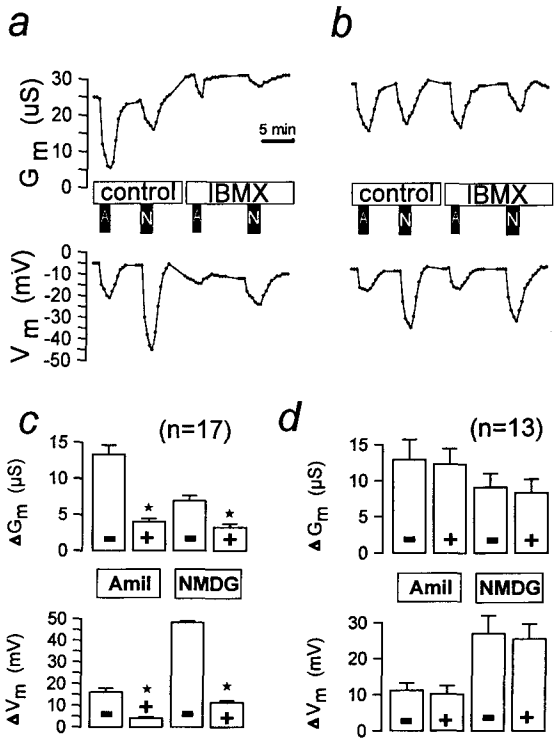

Fig. 2. Coexpression of αβγ-rENaC with either wild type CFTR (a,c) or G551D-CFTR (b,d). a: Typical conductance (G_m, upper part) and voltage (V_m, lower part) recordings before (control) and after stimulation of the oocyte with IBMX (1mmol/l). Application of 5 μmol/l amiloride (A) or replacement of extracellular Na^+ by *N*-methyl-D-glucamine (N) inhibited G_m and hyperpolarized V_m, respectively. Effects of amiloride and *N*-methyl-D-glucamine were attenuated in the presence of IBMX. c: Summary of the experiments as shown in a: Inhibition of G_m and hyperpolarization of V_m by either amiloride (Amil) or *N*-methyl-D-glucamine (NMDG) were significantly reduced after stimulation with 1 mmol/l IBMX (+) and when compared to recordings in the absence of IBMX (−). b,d: No inhibition of amiloride sensitive Na^+ currents by IBMX could be detected when αβγ-rENaC was coexpressed together with mutant G551D-CFTR: Effects of amiloride and NMDG on whole cell Na^+ conductance and membrane voltage, respectively, were independent of the presence of IBMX.

from a previous study [8] suggest that both proteins may colocalize in close proximity at the plasma membrane. They may be either functionally coupled via unknown additional proteins or crosstalk via direct physical interaction.

We examined the latter possibility using yeast two hybrid analysis [5]. This assay is based on the fact that the DNA binding (BD) and the activation (AD) domains of the yeast Gal4p transcriptional activator can be independently fused to heterologous proteins and, if these other proteins interact upon coexpression of the hybrids in yeast, then transcription of a reporter gene(s) is activated. Therefore, we fused various CFTR-cDNA fragments coding for N or C termini, the four intracellular (cytosolic) loops, the R-domain (R), the region between TM6 and NBF1 (PNBF1), and NBF1 and NBF2 to either the BD or the AD. Similarly, α-, β- and γ-subunits of rENaC were fused to either AD or BD.

Cotransformation of α-rENaC with the CFTR fragment containing the pre-NBF1, NBF1, and R domains (P1-R, amino acids 351–830) generated transformants that were positive for both colony growth on media lacking histidine and for colony staining in the X-gal filter lift assay. Positive two-hybrid results were seen irrespective of the fusion partner of the α-subunit, i.e. both the α-rENaC-AD/P1-R CFTR-BD and the α-rENaC-BD/P1-R CFTR-AD combinations indicated protein-protein interaction by two hybrid analysis. Notably, all single plasmid transformants were negative for reporter gene activity. Transformation of two different yeast strains, HF7c and SFY526, showed similar results. These results suggests direct interaction of α-rENaC and the cytoplasmic P1-R domain of CFTR. Two hybrid analysis of other combinations, e.g. coexpression of α-ENaC with other intracellular parts of CFTR such as intracellular loops, N and C termini or NBF2, did not indicate any interaction with α-rENC (Table 1). Moreover, R and NBF1 domains coexpressed separately with α-rENaC gave negative results, suggesting that interaction requires the whole P1-R domain or that smaller domains not defined here, may support an interaction with α-rENaC. Both N and C termini of α-rENaC are located in the cytosol and may be important for interaction with CFTR. We

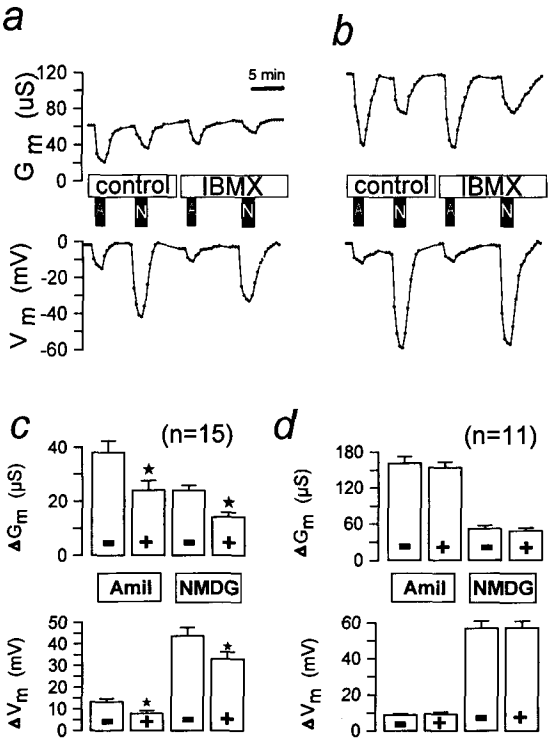

Fig. 3. Coexpression of αβγ-rENaC with CFTR fragments comprising mainly the first nucleotide binding fold and R-domain (Pl-R, amino acids 394–830). Pl-R fragments were derived from either wild type CFTR (a,c) or a G551D-CFTR mutant (b,d). a: Typical conductance (G_m, upper part) and voltage (V_m, lower part) recordings before (control) and after stimulation of the oocyte with IBMX (1 mmol/l). Application of 5 μmol/l amiloride (A) or replacement of extracellular Na^+ by *N*-methyl-D-glucamine (N) inhibited G_m and hyperpolarized V_m, respectively. Effects of amiloride and *N*-methyl-D-glucamine were attenuated in the presence of IBMX. c: Summary of the experiments as shown in a: Inhibition of G_m and hyperpolarization of V_m by either amiloride or *N*-methyl-D-glucamine (NMDG) were significantly reduced after stimulation with 1 mmol/l IBMX (+) and when compared to recordings in the absence of IBMX (−). b,d: No inhibition of amiloride sensitive Na^+ currents by IBMX could be detected when αβγ-rENaC was coexpressed together with Pl-R fragments of mutant G551D-CFTR: Effects of amiloride and NMDG on whole cell Na^+ conductance and membrane voltage, respectively, were independent of the presence of IBMX.

therefore fused both N (α-NT, amino acids 1–110) and C termini (α-CT, amino acids 612–700) to the activation domain. Positive results (colony growth on his^- plates, colony stain in the X-gal filter lift assay) were obtained for α-CT but not for α-NT when coexpressed with the Pl-R domain. All other combinations were negative (Table 1). These data are in agreement with the results from coexpression in oocytes and suggest that downregulation of rENaC by CFTR is due to interaction of the C terminus of α-rENaC with the Pl-R domain of CFTR. This hypothesis was further supported by the fact that the same mutation in the NBF1 region (G551D) that eliminated CFTR dependent downregulation of rENaC also abolished protein-protein interaction detected by the two hybrid analysis (Table 1). Lack of an interaction of G551D/Pl-R with α-rENaC may explain the inability of the G551D/Pl-R CFTR fragment to inhibit ENaC currents in oocytes.

These findings confirm previous observations that CFTR, when stimulated by cAMP enhancing agonists, inhibits epithelial Na^+ conductance. This observation was made first in recombinant expression systems [13] and the physiological significance was confirmed in kidney and colonic crypt cells [4,9]. Recently, similar observations were also made in respiratory epithelial cells (unpublished from the author's laboratory). Therefore we suggest that the interactions observed are of physiological relevance and may help to understand the molecular mechanisms underlying the enhanced Na^+ transport and the disturbed salt and water balance of the airways in cystic fibrosis patients. The data presented here do not ultimately prove direct interaction of CFTR and ENaC as the key mechanism for CFTR dependent downregulation of ENaC. However, the results from two hybrid analysis make protein-protein interaction very likely. Coimmunoprecipitation experiments are on their way in order to examine whether activated CFTR directly inhibits ENaC.

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