Wild type but not ΔF508 CFTR inhibits Na^+ conductance when coexpressed in *Xenopus* oocytes

M. Mall, A. Hipper, R. Greger*, K. Kunzelmann

Physiologisches Institut der Albert-Ludwigs-Universtität Freiburg, Hermann-Herder-Str. 7, D-79104 Freiburg, Germany

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Abstract **Airway epithelial cells bearing mutations of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) possess an increased Na^+ conductance along with their well described defect of cAMP dependent Cl^- conductance. Currently it is not clear, how this occurs, and whether it is due to a CFTR control of epithelial Na^+ conductances which might be defective in CF patients. In the present study, we have tried to identify possible interactions between both CFTR and the epithelial Na^+ conductance by overexpressing respective cRNAs in *Xenopus* oocytes. The expression of all three (α, β, γ) subunits of the rat epithelial Na^+ channel (rENaC) and wild type (wt) CFTR resulted in the expected amiloride sensitive Na^+ and IBMX (1 mmol/l) activated Cl^- currents, respectively. The amiloride sensitive Na^+ conductance was, however, inhibited when the wt-CFTR Cl^- conductance was activated by phosphodiesterase inhibition (IBMX). In contrast, IBMX had no such effect in ΔF508 and Na^+ channels coexpressing oocytes. These results suggest that wt-CFTR, but not ΔF508-CFTR, is a cAMP dependent downregulator of epithelial Na^+ channels. This may explain the higher Na^+ conductance observed in airway epithelial cells of CF patients.**

Key words: Cystic fibrosis; CFTR; Na^+ absorption; Cl^- conductance; Cl^- secretion; Protein kinase A

1. Introduction

Cystic fibrosis (CF) is characterized by defective cAMP dependent Cl^- conductance and increased Na^+ conductance in epithelial cells [3,15]. The defect in cAMP dependent activation of Cl^- conductance in epithelial cells is explained by mutations of the CFTR protein. According to previous reports, CFTR forms a Cl^- channel [2,7,15]. However, it has become clear now that CFTR functions not only as a Cl^- channel but also controls exocytosis [4,10] and acts as a more general conductance regulator [7,11,19]. Increased Na^+ conductance in CF was found in transepithelial measurements [3] and was recently confirmed in patch clamp studies [12]. These results suggest that mutations in CFTR protein somehow enhance the amiloride sensitive Na^+ conductance. A recent unpaired study indeed shows downregulation by cAMP of Na^+ currents in MDCK cells and fibroblasts expressing CFTR [19]. In the present study, we examined possible interactions of both wt-CFTR and ΔF508 and rENaC in the *Xenopus* oocyte expression system.

*Corresponding author. Fax: (49) (761) 203-5191.

2. Materials and methods

2.1. CFTR-cRNA and ΔF508-cRNA, cRNAs of the epithelial rat Na^+ channel (rENaC)

A 4.7 kb sequence encoding CFTR was subcloned into p-Bluescript vector (Stratagene) using the restriction sites *Kpn*I and *Not*I and amplified in *E. coli* (XL1-Blue, Stratagene) [9]. For in vitro transcription of cRNA the plasmid was linearized with *Kpn*I and cRNA was synthesized using T7 promotor with the respective polymerase and a 5′ cap (mCAP mRNA capping kit, Stratagene). The CFTR mutation ΔF508 was produced by subcloning the 4.7 kb fragment containing CFTR into p-alter vector (Altered Sites in vitro Mutagenesis System, Promega, Heidelberg, Germany). Single stranded cDNA was obtained by helper phage R 408. Synthesis of mutated CFTR-cDNA was induced by annealing of ampicillin repair oligonucleotide and oligonucleotide primer carrying the deletion mutation ΔF508. Correct mutation was confirmed by using the PRISM cycle sequencing kit (Perkin Elmer) and an automated sequencer (Pharmacia, Germany). The three (α, β, γ) subunits of the epithelial Na^+ channel of rat amiloride sensitive Na^+ channel (rENaC, kindly provided by Prof. Dr. B. Rossier, Pharmacological Institute of Lausanne, Switzerland) were subcloned into p-Bluescript, linearized with *Not*I and in vitro transcribed using T7 promotor and polymerase.

2.2. Electrophysiological analysis of Xenopus oocytes

The methods to record voltages and currents of *Xenopus* oocytes have been described in previous reports [9]. In brief, adult *Xenopus laevis* female frogs were obtained from H. Kähler (Bedarf für Entwicklungsbiologie, Hamburg, Germany). After isolation, oocytes were dispersed and defolliculated by 1 h treatment with collagenase (type A, Boehringer). Subsequently, oocytes were rinsed 10 times and kept in a Na^+-HEPES buffer (pH 7.55), supplemented with pyruvate (2.5 mM), theophylline (0.5 mM) and gentamicin (50 mg/l) at 14–18°C. Oocytes of identical batches were injected each with 10–50 ng of cRNA dissolved in about 50 nl ddH_2O (PV830 pneumatic pico pump, WPI, Germany). Water injected oocytes served as controls.

2–4 days after injection oocytes were impaled with two electrodes (Clark instruments) which had resistances of ≤1 MΩ when filled with 2.7 mol/l KCl. A flowing (2.7 mol/l) KCl electrode served as a bath reference. The membrane currents were measured by voltage clamping of the oocytes (OOC-1, WPI, Germany) from −80 to +40 mV in steps of 10 mV and conductances were calculated following Ohm's law.

All used compounds were of highest available grade of purity. They were obtained from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany). Data are presented as original recordings and as mean values ± SEM (n = number of observations). Statistical analysis was performed according to Students t test. P values < 0.05 were accepted to indicate statistical significance.

3. Results

3.1. The properties of rENaC in Xenopus oocytes

Fig. 1A shows the current voltage (I/V) curve of a *Xenopus* oocyte injected with rENaC-cRNA. It is obvious that a large fraction of the current can be inhibited by a low concentration of amiloride (10 μmol/l). In a series of 21 similar experiments the amiloride effect was as follows: the zero current voltage (V_m) was hyperpolarized significantly from -6.7 ± 2.5 to -32 ± 3 mV and the conductance (G_m) was reduced sig-

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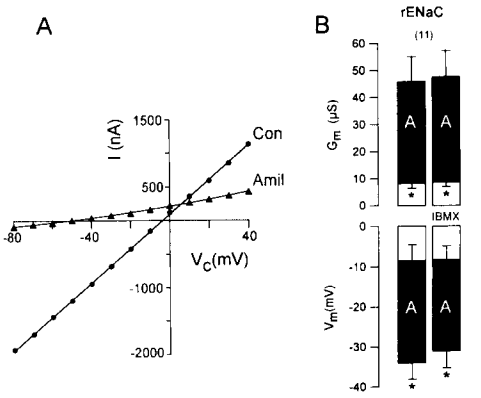

Fig. 1. Expression of rENaC in *Xenopus* oocytes. (A) Current voltage curves in the absence (con) and presence of amiloride (Amil, 10 μmol/l). (B) Summary of measurements in the absence (Con) and presence of IBMX (1 mmol/l). Mean values ± S.E.M. (number of observations). 'A' and black bars denote the effect of amiloride (10 μmol/l) on conductance (G_m) and zero current voltage (V_m). * = significant difference amiloride versus control.

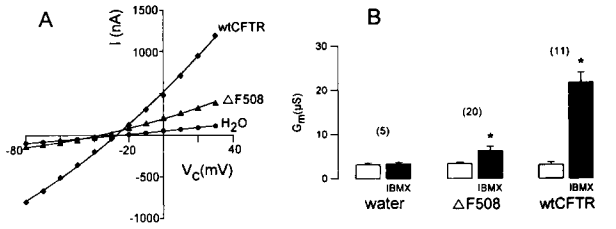

Fig. 2. Expression of CFTR in *Xenopus* oocytes. (A) Current voltage curves obtained in three oocytes incubated by IBMX (1 mmol/l). The lowest curve corresponds to a water (H_2O) injected oocyte; the middle curve was obtained in an oocyte injected with ΔF508; and the upper curve was obtained in a wt-CFTR injected oocyte. (B) Summary of measurements in all three series, effect of IBMX (1 mmol/l). Mean values ± S.E.M. (number of observations). G_m, membrane conductance. Water, water injection; ΔF508, ΔF508-CFTR injected oocytes; wtCFTR, wild-type CFTR injected oocytes. *, significant difference IBMX versus control.

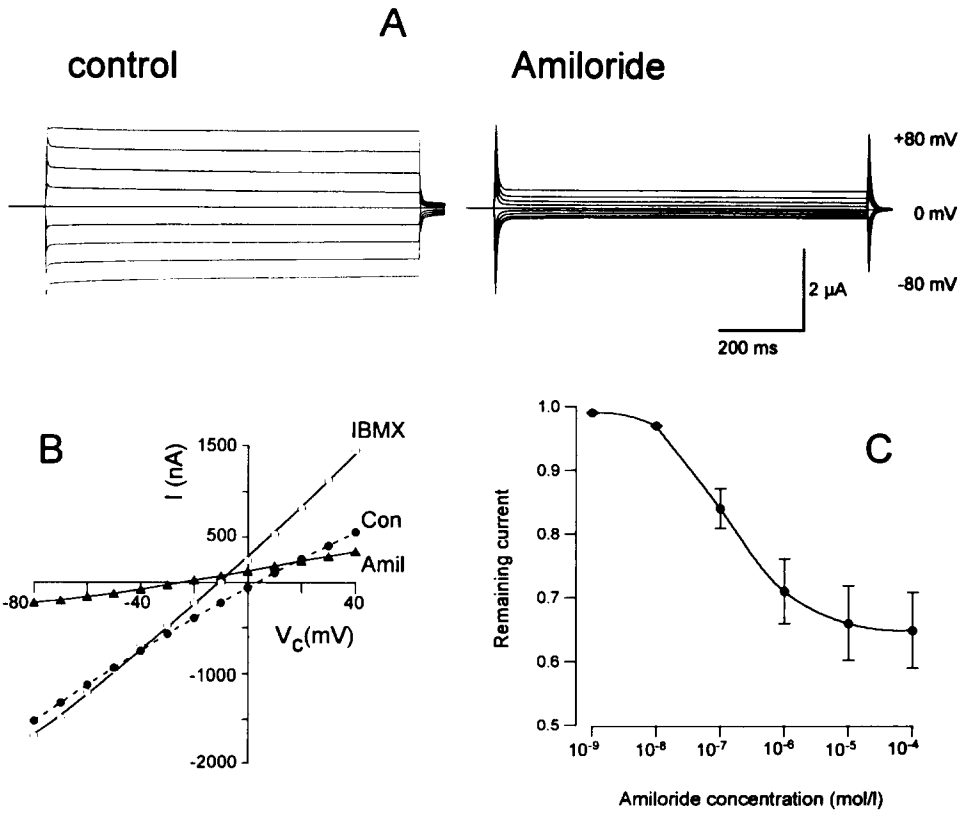

Fig. 3. Expression of wt-CFTR plus rENaC in *Xenopus* oocytes. (A) Typical voltage clamp experiment; effect of amiloride (10 μmol/l). The membrane current for the individual clamp voltages between −80 and +80 mV is shown in the absence (control) and presence of amiloride. Note the strong inhibition of the current by amiloride. (B) Current voltage (I/V) curves obtained in one oocyte coinjected with wt-CFTR plus rENaC. Con, control I/V curve; IBMX, I/V curve after addition of IBMX (1 mmol/l); Amil, I/V curve in the presence of amiloride (10 μmol/l). Note the increase in conductance by IBMX and the reduction in conductance and hyperpolarization induced by amiloride. (C) Concentration response curve for amiloride in wt-CFTR plus rENaC coinjected oocytes in the presence of IBMX (1 mmol/l) ($n = 5$). Data are given as mean (± S.E.M.) of remaining current as a function of the amiloride concentration. Note that the IC_{50} is approximately 0.2 μmol/l, which is typical for the high affinity amiloride inhibitable Na^+ channel [5]. The remaining current in these IBMX activated oocytes of 63% is carried mostly by Cl^-

nificantly from 34 ± 6.2 to 7.6 ± 2.0 μS. In another series of 11 experiments the effect of amiloride (10 μmol/l) was tested in the absence and presence of isobutylxanthine (IBMX, 1 mmol/l, >5 min). This was done in order to test whether rENaC was upregulated by cAMP and PKA-dependent phosphorylation. The results are summarized in Fig. 1B. It is evident that amiloride had a strong and comparable effect in the absence and presence of IBMX. Furthermore, it is evident that G_m and V_m were not altered by IBMX. In water injected oocytes neither V_m (−38 ± 2.1 versus −37 ± 2.1, $n = 8$) nor G_m (2.5 ± 0.4 versus 2.6 ± 0.4, $n = 8$) were altered by amiloride.

3.2. The properties of wt-CFTR and Δ508 in Xenopus oocytes

In Fig. 2A the I/V curves of 3 oocytes are compared. It is evident that water injected and IBMX treated oocytes have a very small G_m. G_m is slightly increased in IBMX treated and ΔF508 injected oocytes. The largest G_m is found in IBMX treated wt-CFTR injected oocytes. In the entire series (Fig. 2B) of water-, ΔF508-, and wt-CFTR-injected oocytes IBMX had no effect on V_m and G_m in the water injected oocytes ($n = 5$). In ΔF508 injected oocytes ($n = 20$) IBMX had no significant effect on V_m (−30 ± 2.5 versus −31 ± 2.0 mV) but G_m was enhanced significantly from 3.4 ± 0.3 to 6.3 ± 1.0 μS. In wt-CFTR injected oocytes IBMX had a slight effect on V_m (−18 ± 3.8 versus −22 ± 3.8 mV, $n = 11$) and increased G_m significantly and strongly from 3.1 ± 0.6 to 21.8 ± 2.3 μS. In another series it was examined whether amiloride (10 μmol/l) had any effect in wt-CFTR injected oocytes ($n = 13$) and it was found that amiloride had no effect on V_m (−30 ± 2.7 versus −29 ± 2.9 mV) and on G_m (9.0 ± 1.0 versus 8.8 ± 1.0 μS).

3.3. Coexpression of wt-CFTR and rENaC

It was expected that oocytes coexpressing wt-CFTR and rENaC should possess both conductances. This is examined in Fig. 3A,B. Amiloride reduced the membrane current markedly. The corresponding I/V curve is shown in Fig. 3B. IBMX (1 mmol/l) enhanced G_m markedly with little change in V_m. Amiloride (10 μmol/l) reduced G_m strongly and shifted V_m to a hyperpolarized value. Therefore, both types of conductances, the IBMX stimulated Cl^-- and the amiloride inhibitable Na^+ conductance were present in these oocytes. The concentration response to amiloride ($n = 5$) was examined next. The results are depicted in Fig. 3C. Amiloride had an apparent IC_{50} of 0.2 μmol/l, which is very similar to published values for this type of conductance [5].

Next we examined whether the IBMX induced current was a Cl^- current. To this end V_m and G_m were measured in the absence and presence of IBMX (1 mmol/l) with Cl^- or gluconate as the major anion in the bath (equimolar replacement of 96 mmol/l Cl^- by gluconate (5 mM Cl^- remaining)) in two types of oocytes, one injected with wt-CFTR plus rENaC and the other with ΔF508 plus rENaC. The results are summarized in Fig. 4A. In wt-CFTR injected oocytes the replacement of Cl^- by gluconate in the absence of IBMX ($n = 16$) had little effect on V_m (−6.8 ± 1.5 versus −3.9 ± 1.7 mV) and reduced G_m slightly but significantly from 16.3 ± 2.5 to 14.8 ± 2.3 μS. Addition of IBMX in the Cl^- solution had a significant hyperpolarizing effect (−5.9 ± 1.4 versus −10.6 ± 1.1 mV) and enhanced G_m significantly from 16.7 ± 2.1 to 46.4 ± 9.2 μS. The replacement of Cl^- by gluconate in the presence of IBMX ($n = 16$) had a strong depolarizing effect (−10.6 ± 1.1 versus 14.6 ± 2.3 mV) and reduced G_m significantly from 46.4 ± 9.2 to 26.4 ± 6.5 μS.

The corresponding data in ΔF508 and rENaC injected oocytes ($n = 9$) are also summarized in Fig. 4A. V_m was unaltered by IBMX and by the replacement of Cl^- by gluconate. In the absence of IBMX G_m was 21.9 ± 6.6 μS and was unaltered in the presence of gluconate (21.0 ± 6.5 μS). Addition

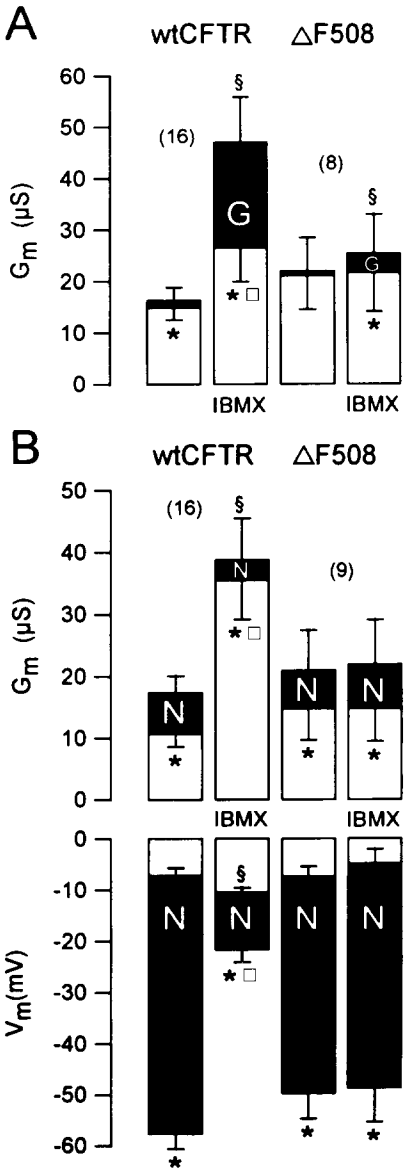

Fig. 4. Expression of wt-CFTR plus rENaC or ΔF508 plus rENaC in *Xenopus* oocytes, effect of IBMX (1 mmol/l). (A) Effect of replacement of Cl^- by gluconate on conductance (G_m). Mean values ± S.E.M. (number of observations). G, gluconate. *, significant difference between Cl^- and gluconate; §, significant effect of IBMX; □, significant difference of Δ (± IBMX). Note that gluconate has little effect in the absence of IBMX and in ΔF508 plus rENaC expressing oocytes. (B) Effect of replacement of Na^+ by *N*-methyl-D-glucamine (NMDG) on conductance (G_m) and voltage (V_m). Mean values ± S.E.M. (number of observations). N, NMDG. *, significant difference between Na^+ and NMDG; §, significant effect of IBMX; □, significant difference of Δ (± IBMX). Note that NMDG has little effect in the presence of IBMX in wt-CFTR plus rENaC coexpressing oocytes. In ΔF508 plus rENaC expressing oocytes the NMDG effects are equally strong in the absence and presence of IBMX.

of IBMX (1 mmol/l) had no effect on V_m (−5.4 ± 2.4 versus −3.6 ± 2.4 mV) but enhanced G_m slightly and significantly to 25.4 ± 7.6 μS. The replacement of Cl^- by gluconate now reduced G_m significantly to 21.6 ± 7.5 μS. These data indicate that IBMX increases a Cl^- conductance in wt-CFTR and has a much smaller effect in ΔF508 injected oocytes. Similar observations were made with 8-chloro-phenyl-thio-cAMP or forskolin, indicating that IBMX acted via cAMP.

3.4. The Na^+ conductance of oocytes coexpressing wt-CFTR, ΔF508 and rENaC

To test for the Na^+ conductance, Na^+ was replaced by *N*-methyl-D-glucamine (NMDG, equimolar replacement) in the bath. The data of both series (coexpression of wt-CFTR plus rENaC, $n = 16$ and ΔF508 plus rENaC, $n = 9$) are summarized in Fig. 4B. In the absence of IBMX Na^+ replacement by NMDG reduced G_m significantly (17.4 ± 2.7 versus 10.7 ± 2.1 μS) and induced a very strong hyperpolarizing effect (−7.2 ± 1.5 versus −58 ± 3.0 mV). This strong hyperpolarization reflects the change in the E^+_{Na} from around +60 to −65 mV. The addition of IBMX (1 mmol/l) enhanced G_m and hyperpolarized V_m significantly (cf. above). Now the replacement of Na^+ by NMDG had a much smaller effect both, on G_m (38.7 ± 6.7 versus 35.5 ± 6.3 μS) and on V_m (−10.4 ± 0.9 versus −21.6 ± 2.4 mV). This suggests that the Na^+ conductance is downregulated when the Cl^- conductance is upregulated by IBMX.

In ΔF508 and rENaC coexpressing oocytes ($n = 9$, Fig. 4B) and in the absence of IBMX replacement of Na^+ by NMDG had a strong effect on G_m (21.0 ± 6.4 versus 14.8 ± 5.1 μS) and V_m (−7.4 ± 1.9 versus −50 ± 5.0 mV). Addition of IBMX (1 mmol/l) had a small albeit significant effect on G_m (cf. above). The effect of Na^+ replacement by NMDG was not changed by IBMX. G_m was reduced significantly from 21.9 ± 7.2 to 14.8 ± 5.3 μS and V_m hyperpolarized from −4.9 ± 2.8 to −49 ± 6.7 mV. These data indicate that IBMX has little effect on the Cl^- conductance of these oocytes and, accordingly, does not change the Na^+ conductance.

3.5. The amiloride effect of oocytes coexpressing wt-CFTR, ΔF508 and rENaC

Next we examined whether the conductance downregulated by IBMX of oocytes coexpressing wt-CFTR and rENaC was in fact the specific epithelial, amiloride sensitive Na^+ conductance. To this end amiloride (10 μmol/l) was examined in these oocytes in the absence and presence of IBMX (1 mmol/l) and the data were compared to oocytes expressing ΔF508 plus rENaC. Two typical experiments are depicted in Fig. 5. It is evident that the amiloride effect on G_m and V_m is sharply attenuated in the presence of IBMX (1 mmol/l) in wt-CFTR plus rENaC but not in ΔF508 plus rENaC coexpressing oocytes. The data are summarized in Fig. 6 for both series. In wt-CFTR plus rENaC coexpressing oocytes in the absence of IBMX ($n = 21$) amiloride (10 μmol/l) reduced G_m significantly from 20.3 ± 2.9 to 7.2 ± 1.2 μS and hyperpolarized strongly from −7.5 ± 1.4 to −24 ± 2.1 mV. The amiloride effect was sharply attenuated in the presence of IBMX (1 mmol/l). G_m fell only slightly (37.3 ± 5.1 versus 33.4 ± 4.8 μS) and V_m was hyperpolarized less from −12 ± 1.1 to −16 ± 1.3 mV. In ΔF508 plus rENaC coexpressing oocytes ($n = 17$), on the other hand, the effect of amiloride was equally strong in the absence and presence of IBMX (ΔG_m 7.9 ± 1.8 versus 9.2 ± 2.2 μS and ΔV_m −22 ± 2.8 versus −21 ± 3.2 mV). These data prove that IBMX reduces the amiloride inhibitable Na^+ conductance in oocytes coexpressing wt-CFTR and rENaC.

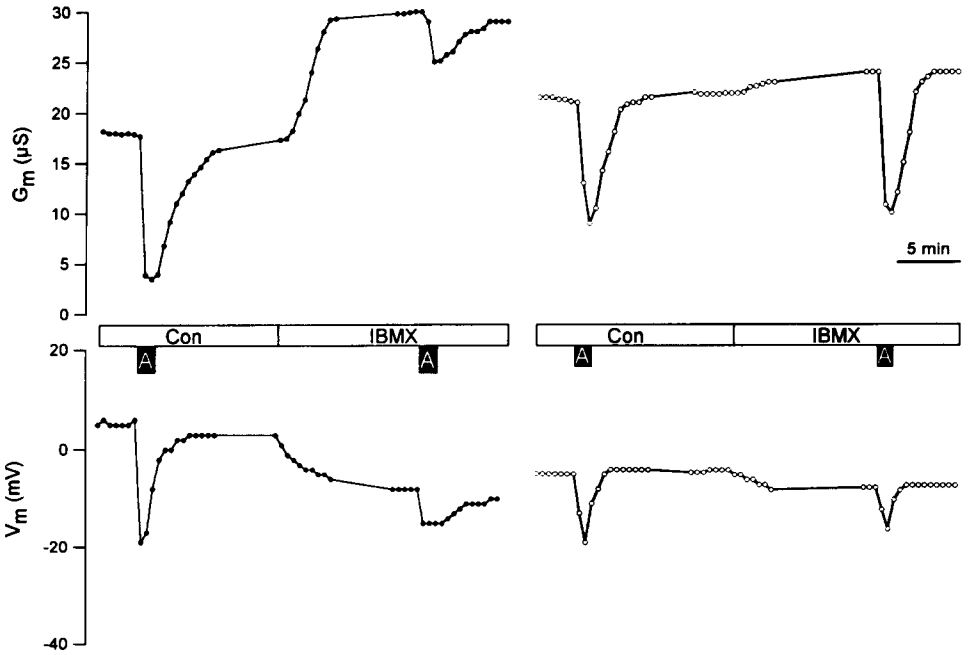

Fig. 5. Expression of wt-CFTR plus rENaC or ΔF508 plus rENaC in *Xenopus* oocytes, effect of IBMX (1 mmol/l) on amiloride (10 μmol/l) inhibition of rENaC. Typical conductance (G_m) and voltage (V_m) recordings in an oocyte coexpressing wt-CFTR plus rENaC (left panel, closed circles) and ΔF508 plus rENaC (right panel, open circles). Con, control; IBMX, addition of 1 mmol/l IBMX to the bath; A, addition of 10 μmol/l amiloride to the bath. Note the attenuation of the amiloride effect in wt-CFTR plus rENaC coexpressing oocytes pretreated with IBMX. IBMX has no effect on the amiloride inhibition in ΔF508 plus rENaC coexpressing oocytes.

4. Discussion

The Na^+ conductance in the airways of patients suffering from CF is enhanced as shown initially by the increased amiloride sensitive transepithelial voltage in the airways of CF patients [3] and confirmed later in patch clamp experiments [12]. According to these results, hyperabsorption of NaCl and water in CF respiratory epithelium was postulated resulting in dehydration of the airways. However, it remained obscure how this enhanced Na^+ conductance correlates to the mutations of the cystic fibrosis transmembrane conductance regulator and the reduced cAMP activated Cl^- conductance which is found in CF [19].

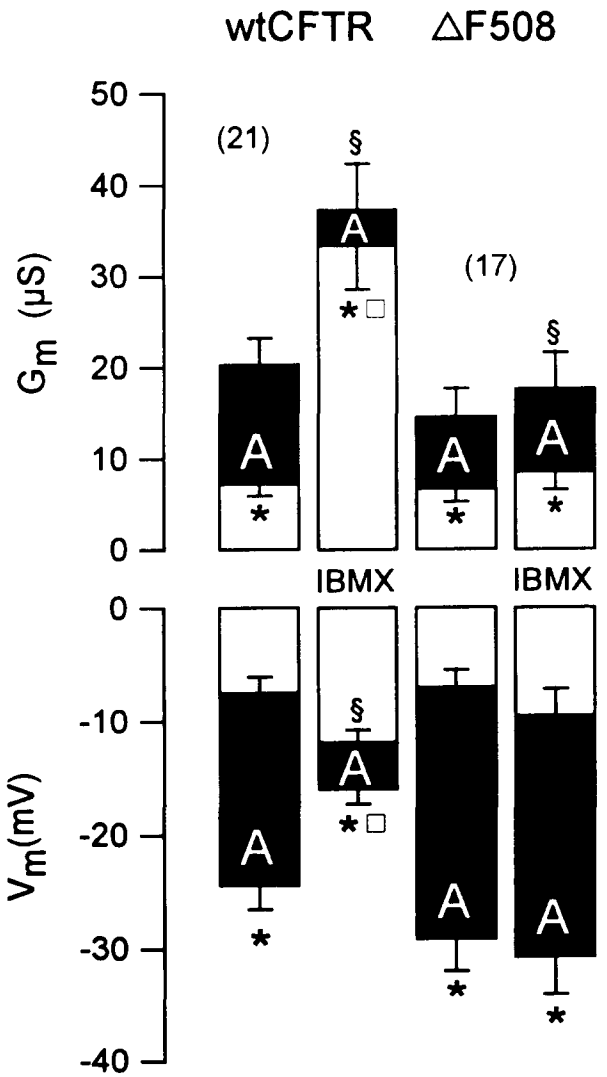

Fig. 6. Expression of wt-CFTR plus rENaC or ΔF508 plus rENaC in *Xenopus* oocytes, effect of IBMX (1 mmol/l) on amiloride (10 μmol/l) inhibition of rENaC. Mean values ± S.E.M., (number of observations) of conductance (G_m) and voltage (V_m) recordings in oocytes coexpressing wt-CFTR plus rENaC (left two columns) and ΔF508 plus rENaC (right two columns). Effect of addition of amiloride (A; 10 μmol/l). *, significant difference caused by amiloride; §, significant effect of IBMX; □, significant difference of Δ (± IBMX). Note that amiloride has little effect in the presence of IBMX in wt-CFTR plus rENaC coexpressing oocytes. In ΔF508 plus rENaC expressing oocytes the amiloride effects are equally strong in the absence and presence of IBMX.

Previous studies suggest that CFTR, besides its effects on cAMP activated Cl^- conductance, might also interfere with Ca^{2+} and swelling induced Cl^- conductances [1]. Very recent reports also suggest an interaction of CFTR with the outwardly rectifying Cl^- channel [17], with the multidrug resistant protein [8], and even ROM-K^+ channels [14]. In addition evidence has accumulated that CFTR may be a regulator of exocytosis [4,10,18]. Another recent study shows that amiloride sensitive Na^+ conductance, when coexpressed with CFTR in MDCK cells and 3T3 fibroblasts, is inhibited by increase in intracellular cAMP [19]. All these data suggest a regulatory function of CFTR on other ion conductances.

Another important argument for a reciprocal regulation of Na^+ and Cl^- channels comes from observations in the colonic crypt, where the same cells, or at least electrically coupled cells, possess Cl^- and Na^+ channels and can be stimulated to absorb Na^+ (Cl^-) or secrete Cl^- (Na^+) [6]. The same may hold for respiratory epithelial cells [13]. It is obvious that coactivation of luminal Cl^- and Na^+ channels facilitates Na^+ absorption but cannot explain Cl^- secretion. One would therefore postulate that Na^+ channels must in some way be inhibited when Cl^- secretion is activated. This is in fact suggested by the present data. In contrast to another recent report [19], the Na^+ conductance itself was not cAMP regulated in our study. Therefore, we were able to examine the interference of CFTR and rENaC in the same oocyte in strictly paired fashion. Our observations go one step further by showing that the coexpression of ΔF508 plus rENaC abolishes this cAMP regulated difference in Na^+ conductance function. Therefore, the enhanced Na^+ absorption in the respiratory epithelial cells of CF patients can be looked at as a malfunction not only of the Cl^- conductance but also as loss of Na^+ channel control. The mechanistic aspect of this protein–protein interaction is subject to further studies.

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