Non-specific activation of the epithelial sodium channel by the CFTR chloride channel

Georg Nagel⁺, Tanjef Szellas, John R. Riordan¹, Thomas Friedrich & Klaus Hartung

Max-Planck-Institute of Biophysics, Kennedyallee 70, D-60596 Frankfurt/M., Germany and ¹S.C. Johnson Medical Research Center, Mayo Clinic, 13400 E. Shea Blvd, Scottsdale, AZ-85259, USA

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The genetic disease cystic fibrosis is caused by mutation of the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR). Controversial studies reported regulation of the epithelial sodium channel (ENaC) by CFTR. We found that uptake of $^{22}\text{Na}^+$ through ENaC is modulated by activation of CFTR in oocytes, coexpressing CFTR and ENaC, depending on extracellular chloride concentration. Furthermore we found that the effect of CFTR activation could be mimicked by other chloride channels. Voltage- and patch-clamp measurements, however, showed neither stimulation nor inhibition of ENaC-mediated conductance by activated CFTR. We conclude that the observed modulation of $^{22}\text{Na}^+$ uptake by activated CFTR is due to the effect of CFTR-mediated chloride conductance on the membrane potential. These findings argue against the notion of a specific influence of CFTR on ENaC and emphasize the chloride channel function of CFTR.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive genetic disease affecting secretion and absorption in epithelia (Riordan, 1993; Pilewski and Frizzell, 1999; Quinton, 1999). Quinton (1983) showed that chloride conductance of sweat ducts is decreased in CF. For airway cells it was postulated by Boucher *et al.* (1988) that in addition to a decreased chloride conductance, an increased sodium conductance also contributes to the CF phenotype. The product of the gene mutated in CF patients, the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan *et al.*, 1989), was found to be a chloride channel, activated by protein phosphorylation and interaction with nucleoside triphosphates (Anderson *et al.*, 1991; Bear *et al.*, 1992; Nagel *et al.*, 1992). Consistent with the observed increase in sodium conductance in CF airways, there have been several

reports that active CFTR inhibits the epithelial sodium channel, ENaC (Stutts *et al.*, 1995; Mall *et al.*, 1996; Briel *et al.*, 1998; Chabot *et al.*, 1999; Ji *et al.*, 2000; Jiang *et al.*, 2000). In contrast, stimulation of ENaC by activation of CFTR in the sweat duct was described recently (Reddy *et al.*, 1999). We have attempted to examine the basis of these apparently opposing findings.

RESULTS

Uptake of $^{22}\text{Na}^+$ in oocytes coexpressing ENaC and CFTR or other chloride channels

In order to test the function of CFTR as a regulator of the epithelial sodium channel (ENaC) we coexpressed CFTR and the α,β,γ subunits of ENaC (Canessa *et al.*, 1993) in oocytes of *Xenopus laevis*. The uptake of $^{22}\text{Na}^+$ was measured in the presence and absence of the inhibitor of ENaC, amiloride.

Amiloride-sensitive $^{22}\text{Na}^+$ uptake was increased by activation of CFTR with the [cAMP] raising agents forskolin and IBMX at physiological (121 mM) extracellular $[\text{Cl}^-]$ (see Figure 1A). Activation of CFTR in a low $[\text{Cl}^-]$ solution (2 mM), however, decreased amiloride-sensitive uptake of $^{22}\text{Na}^+$. Specific uptake was not influenced by the extracellular chloride concentration if CFTR was not activated (data not shown). Addition of forskolin and IBMX had no significant effect on amiloride-sensitive $^{22}\text{Na}^+$ uptake at either 121 mM or 2 mM extracellular $[\text{Cl}^-]$ if only ENaC (without CFTR) was expressed ($n = 3$, data not shown).

We then tested the effect of coexpressing another chloride channel, ClC-0 (Jentsch *et al.*, 1990), which shares no homology with CFTR. ClC-0 mediates large chloride conductances, comparable to CFTR, and its apparent open probability is large enough in both high and low $[\text{Cl}^-]$ within the voltage range of

⁺Corresponding author. Tel: +49 69 6303 303; Fax: +49 69 6303 420; E-mail: Georg.Nagel@mpibp-frankfurt.mpg.de

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Fig. 1. Dependence of amiloride-sensitive $^{22}\text{Na}^+$ uptake on chloride conductance and concentration. **(A)** CFTR/ENaC coexpressing oocytes in different solutions: 1, 121 mM Cl^- , CFTR not activated; 2, 121 mM Cl^- , CFTR activated; 3, low (2 mM) Cl^- , CFTR activated. **(B)** ClC-0/ENaC coexpressing oocytes in different solutions: 4, 121 mM Cl^- ; 5, low (2 mM) Cl^- . (Normalized mean values $\pm$ SE from five experiments (=oocyte batches with >50 oocytes per column).

interest (Pusch *et al.*, 1995). Amiloride-sensitive $^{22}\text{Na}^+$ uptake was enhanced by coexpression of ClC-0 in normal (121 mM) chloride concentration, whereas it was inhibited in a low $[\text{Cl}^-]$ solution (2 mM), see Figure 1B. The membrane potential is expected to change with increased chloride conductance depending on external chloride concentration, which we confirmed in current-clamp experiments on CFTR-ENaC-coexpressing oocytes, e.g. activation of ENaC resulted in a membrane potential of +6 mV, which changed to -14 mV in normal chloride and to +20 mV in a low $[\text{Cl}^-]$ solution when both ENaC and CFTR were activated. Therefore, these results may reflect the influence of membrane potential on sodium uptake and argue against strong inhibition of ENaC by activated CFTR as suggested previously for coexpressing oocytes.

Effect of CFTR on ENaC-induced electrical current in voltage-clamped oocytes

Next we examined the effect of CFTR on ENaC under voltage-clamp conditions in CFTR-ENaC-coexpressing oocytes. It was noticeable that the ENaC-induced, as well as the CFTR-induced, conductances were considerably higher than those reported by others using similar or higher amounts of injected c-RNA and reporting inhibition of ENaC by CFTR (Mall *et al.*, 1996; Briel *et al.*, 1998; Chabot *et al.*, 1999; Ji *et al.*, 2000; Jiang *et al.*, 2000). We included in our study oocytes expressing high ENaC- and CFTR-specific conductances because it was shown previously that inhibition of ENaC by activated CFTR was dependent on CFTR-induced conductance [~90% inhibition at 100 μS , ~50% inhibition at 30 μS (Briel *et al.*, 1998)]. We also took care that CFTR-mediated conductance equalled or exceeded ENaC-mediated conductance in order to avoid limitation of ENaC regulation by insufficient CFTR expression. Although it was shown that currents larger than ~20 μA are likely to be distorted when obtained with the two-electrode voltage clamp (Baumgartner *et al.*, 1999), we analyzed the influence of CFTR

stimulation on the amiloride-sensitive conductance (ASC) also in oocytes showing such high functional expression (but at injected RNA amounts which were smaller than the previously reported ones). Figure 2A shows a typical example of current-voltage relationships obtained with and without amiloride, before and during CFTR-activation. Figure 2B shows that the amiloride-sensitive conductance (i.e. ENaC) is negligibly influenced by activation of CFTR. In repeated experiments ($n = 15$), using different oocyte batches ($n = 5$) and expression levels (with conductances for ENaC from 20 μS to 500 μS), ASC often changed slightly during the course of the experiment. Amiloride-sensitive conductance increased or decreased up to 30% during ~45 min of CFTR activation and complete deactivation, but we never observed a significant (t -test, 5% level) CFTR-attributable modulation.

Effect of CFTR when using only one-bath-electrode

The lack of influence of activated CFTR on ENaC-activity under voltage-clamp conditions was somewhat surprising because of previous reports from different research groups (Mall *et al.*, 1996; Briel *et al.*, 1998; Chabot *et al.*, 1999; Ji *et al.*, 2000; Jiang *et al.*, 2000), which showed that activation of CFTR inhibits ENaC, when coexpressed in oocytes. Therefore, we investigated the possibility that an uncompensated series resistance might have led to this conclusion.

When determining membrane conductance the potentially error-introducing contribution of an unavoidable series resistance (R_s , i.e. the resistance of the saline between the voltage sensing electrodes) may only be neglected if its magnitude is negligible (e.g. <5%) in relation to the membrane resistance (R_m = the reciprocal of membrane conductance). The resistance that is determined in two-electrode-voltage-clamp experiments is in reality the sum of R_m and R_s which may be called R_t . As long as the series resistance is small compared to the membrane resistance we get: $R_t = R_m + R_s \approx R_m$ and changes in R_m will be faithfully reflected by measuring R_t . If, however, the magnitude of R_m is of the same order as R_s , a change of R_m will have a smaller proportional effect on R_t . The CFTR-mediated membrane conductance in Figure 2A for example, can be estimated to ~250 μS , i.e. 4 k Ω . Variations of this conductance can, therefore, still be measured with a series resistance of 100 Ω but are completely compromised by a series resistance of 20 k Ω which leads to a R_t of 24 k Ω . If this value was considered to reflect R_m , an apparent 'CFTR-induced membrane conductance' of ~40 μS would be obtained which could be maximally increased (by complete 'membrane permeabilization', i.e. $R_m = 0$) to 50 μS . For the CFTR-induced membrane resistance of Figure 2A, a series resistance of 1 k Ω will decrease the apparent CFTR-conductance to 200 μS . When using a two-bath-electrode configuration the extracellular series resistance is dependent upon the distance of the voltage reference electrode to the plasma membrane. In the experiments described above we placed the electrode in close proximity to the oocyte and estimated an extracellular series resistance of ~100 Ω . The series resistance could be increased drastically by placing the voltage reference electrode more distant from the oocyte (data not shown).

Fig. 2. Current–voltage relationship (I–V) of voltage-clamped CFTR/ENaC coexpressing oocytes. **(A)** I–V with (open triangle, open circle) and without (closed triangle, closed circle) amiloride, before (triangles) and during (circles) stimulation of CFTR. **(B)** amiloride-sensitive conductance (ASC) of currents in (A), before (1), during (2) and after (3) stimulation of CFTR. **(C)** same experiment as in (A), but recorded in the one-bath-electrode configuration (with a series resistance of 20 k Ω): I–V with (open triangle, open circle) and without (closed triangle, closed circle) amiloride, before (triangles) and during (circles) stimulation of CFTR. **(D)** Apparent amiloride-sensitive conductance (ASC) of currents in (C), before (1), during (2), and after (3) stimulation of CFTR, notice different scaling! [(A–C) are from one representative experiment, 3 ng CFTR-RNA, 3 $\times$ 8 ng ENaC-RNA, 2 days after injection].

Consideration of the series resistance is even more important when recording in the ‘one-bath-electrode’ configuration, i.e. one electrode is used for voltage sensing and current measurement, and using a ‘saline bridge’ (e.g. a ‘flowing KCl electrode’ as in previous reports, see Mall *et al.*, 1996; Briel *et al.*, 1998). The one-bath-electrode configuration is the only possibility for some commercially available voltage-clamp amplifiers (such as OOC-1 from WPI, used in some previous studies, which showed inhibition of ENaC by activated CFTR; see Mall *et al.*, 1996; Briel *et al.*, 1998). In this case the resistance of the saline bridge and Ag/AgCl-electrode will add to the series resistance, which therefore easily exceeds 1 k Ω . It is therefore conceivable that a CFTR-mediated high membrane conductance led to conditions where membrane conductance modulation by amiloride has little effect on the overall conductance (erroneously considered to reflect membrane conductance). Unfortunately the series resistance was not estimated or even mentioned in any of the previous publications reporting inhibition of ENaC by CFTR.

To test our hypothesis we also performed the experiments in a one-bath-electrode configuration, in this case with a series resistance of ~20 k Ω (see Methods). Figure 2C shows that in the

one-bath-electrode configuration the recorded apparent ‘membrane conductances’ of ENaC and CFTR became smaller, more similar to the ones reported previously. The data of Figure 2C derive from the same experiment as shown in Figure 2A but were obtained with a ‘one-bath-electrode’ configuration introducing an uncompensated series resistance of ~20 k Ω and are, therefore, seriously flawed. Figure 2D shows that now the ENaC-induced conductance seems strongly reduced during CFTR activation. Because the switch between a two-bath-electrodes and a one-bath-electrode configuration was performed rapidly and reversibly we tested the effect of activated CFTR on ENaC in several oocytes ($n = 11$) from different batches ($n = 4$) with both methods. We observed significant (t -test, 5% level) apparent ‘inhibition’ of >50% in every experiment with the one-bath-electrode configuration, whereas no inhibition was observed in the two-bath-electrode mode. Experiments with different saline bridges (4 to 20 k Ω) showed that the extent of the apparent CFTR-mediated inhibition is modulated by the ratio of the resistances of the saline bridge and of the oocyte membrane (data not shown).

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Effect of CFTR on ENaC-induced electrical current in excised plasma membrane patches

To test the effect of activated CFTR on ENaC further, we measured electrical current from giant excised patches (Hilgemann and Lu, 1998; Weinreich *et al.*, 1999) of plasma membrane from oocytes coexpressing CFTR and ENaC. As these membrane patches were 'inside-out', i.e. exposing the cytoplasmic side to the bath solution, and because amiloride inhibits ENaC only from the extracellular side, we measured ENaC-activity by switching between Na⁺-free and Na⁺-containing cytoplasmic saline. CFTR was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (PKA) as previously described (Anderson *et al.*, 1991; Nagel *et al.*, 1992; Weinreich *et al.*, 1999). Thereafter CFTR could be reversibly activated and deactivated in the absence of PKA, simply by applying or withdrawing Mg-ATP. Addition of PKA and phosphorylation of CFTR had no effect on the size of the ENaC-induced current (data not shown). Figure 3A demonstrates separate and simultaneous activation of ENaC- and CFTR-induced electrical current at 0 mV. Figure 3B shows the I-V relationships of these currents in comparison with the sum of the I-Vs of ENaC and CFTR, demonstrating a lack of influence of CFTR on ENaC. When experiments were repeated using different oocyte batches ($n = 5$), neither activation nor inhibition of ENaC by activated CFTR was observed ($n = 16$), see Figure 3C.

DISCUSSION

Herein we report electrochemical coupling between heterologously expressed CFTR and ENaC and lacking evidence for specific regulation of ENaC by activated CFTR.

We therefore confirm the result of a recent report on endogenously expressed CFTR and ENaC (Reddy *et al.*, 1999), which stated that activated CFTR stimulates sodium absorption by ENaC. To reconcile previous reports of CFTR-mediated ENaC-inhibition with the study of Reddy *et al.* (1999) it was suggested (Greger, 2000) that a tissue-specific expression of an additional factor might determine whether CFTR activates or inhibits ENaC. We present a simpler explanation for the observed activation: ENaC is not directly regulated by CFTR but by the membrane potential which changes upon activation of CFTR. This indirect effect of the altered driving force for sodium ions as a consequence of increased chloride conductance in the same membrane may be termed electrochemical coupling.

Reasons for the previously reported controversial findings

It is interesting to note that the reported stimulation of ENaC by activated CFTR (Reddy *et al.*, 1999) was found in current-clamp experiments under conditions of a strong NaCl gradient, which must lead, according to the Goldman-Hodgkin-Katz equation (Hille, 1992), to a voltage-dependent sodium conductance. Electrochemical coupling predicts that activation of CFTR then leads to a change in membrane potential and increased Na⁺ conductance, as indeed found by Reddy *et al.* (1999).

However, electrochemical coupling cannot explain the previously reported inhibition of ENaC by activated CFTR, found in

Fig. 3. Lack of influence of activated CFTR on ENaC in an excised inside-out giant patch. (A) recording of electrical current from a giant patch at 0 mV from an oocyte coexpressing CFTR and ENaC. Activation of ENaC (by Na), then CFTR (by ATP), then CFTR and ENaC simultaneously (by Na + ATP), then CFTR, as indicated. Vertical current deflections result from voltage pulses. (B) Voltage-dependence of CFTR (crosses) and ENaC (pluses) currents, activated separately and simultaneously (diamonds) [from pulses in (A)]. For comparison the sum (stars) of individual CFTR and ENaC currents and the current in absence of Na and ATP (open circles) are also shown. (C) Normalized sodium current ($\pm$ SE) from 16 different giant patches in the absence and presence of ATP (normalized to total current in presence of Na⁺ and ATP, $I_t = I_{CFTR} + I_{ENaC} = 1$).

voltage-clamp studies. We provide evidence, from voltage-clamp and patch-clamp experiments, that in oocytes activation of CFTR does not influence ENaC if the membrane potential is carefully controlled. It was reported previously that the extent of

the inhibition of ENaC by activated CFTR is increased by increasing CFTR-induced conductance (Briel *et al.*, 1998). To ascertain our 'negative result' we investigated CFTR–ENaC interaction over a wide range of expression levels, including CFTR- and ENaC-mediated conductances of significantly higher magnitude than previously reported in studies 'showing inhibition' of ENaC by activated CFTR. Still we could not detect inhibition of ENaC by activated CFTR. But we found a reasonable explanation for the postulated inhibition and its dependence on CFTR-mediated conductance. We show that in the one-bath-electrode configuration a large part of the voltage drop may be across the series resistance instead of the oocyte membrane.

We assume that the reported inhibition of ENaC by activation of CFTR (when coexpressed in oocytes) is due to recordings from oocytes with dramatically decreased membrane resistance (demonstrated in Figure 2A) masked by a series resistance of several k Ω . The apparent unresponsiveness of the measured resistance to application or withdrawal of amiloride then may suggest reversible inhibition of ENaC by activation of CFTR to the unsuspecting observer. It seems likely that a relatively large series resistance could also explain a previously reported correlation between CFTR-mediated chloride conductance and inhibition of ENaC (Briel *et al.*, 1998; Chabot *et al.*, 1999), as well as the correlation between CFTR-mediated chloride conductance and inhibition of the oocyte-endogenous Ca-activated chloride conductance (Kunzelmann *et al.*, 1997).

Our explanation for the reported inhibition of ENaC by activated CFTR in coexpressing oocytes is not limited to one-bath-electrode experiments. As outlined above, the series resistance could be too large for a highly conductive membrane when measuring with two-bath-electrodes if the voltage sensing reference electrode is not placed in close enough proximity to the oocyte. Further experiments, especially in mammalian cells where a similar, though weaker, inhibition was observed (Stutts *et al.*, 1995), are necessary before specific regulation of ENaC by CFTR can be ruled out.

CONCLUSION

The mechanism whereby the lack of active CFTR leads to airway disease is not yet clear (Wine, 1999). Our present results may serve to refocus emphasis on the primary chloride channel function of the protein, and on the question of whether airway pathogenesis is mainly due to reduced secretion of fluid (Ballard *et al.*, 1999) or reduced reabsorption of salt (Zabner *et al.*, 1998).

METHODS

Oocytes, oocyte Ringer's solution (ORi) and CFTR-cRNA were prepared as previously described (Weinreich *et al.*, 1997, 1999). Aspartate replaced all but 2 mM Cl[−] in low Cl[−] ORi. cRNA for CIC-0 (Pusch *et al.*, 1995), a kind gift of Professor T. Jentsch (ZMNH Hamburg), and rat ENaC α,β,γ subunit (Canessa *et al.*, 1993), a kind gift of Professor B. Rossier (University of Lausanne, Switzerland) were prepared as described. For ²²Na⁺ uptake measurements, 5–7 oocytes per test solution were incubated for 5 or 10 min in the presence or absence of amiloride, washed, then separately lysed with SDS and counted in a scintillation counter, as previously described (Vasilets *et al.*, 1991). The mean values were calculated and normalized to amiloride-

sensitive uptake without activation of CFTR. Normalized values from five experiments were averaged for Figure 1. For two-electrode voltage clamping a turbo-tec 05 amplifier (npi electronic, D-71732 Tamm, Germany) was used. The agar bridge (3 M KCl) to the ground electrode had a resistance of ~20 k Ω , the flowing 3 M KCl bridge to the potential reference electrode a resistance of ~200 k Ω . For this 'two-bath-electrode' configuration we estimated a series resistance in the bath of ~100 Ω . The bath electrodes for voltage reference and ground were reversibly connected electrically to record in a 'one-bath-electrode' configuration with a series resistance of ~20 k Ω . This allowed quick switching between the two configurations. CFTR was activated by 0.5 mM IBMX and 10 μ M forskolin. Giant patch experiments and solutions were as previously described (Weinreich *et al.*, 1999, 1997), the main cation being either Na⁺ or N-methyl-D-glucamine⁺.

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