Cystic Fibrosis Transmembrane Conductance Regulator-dependent Up-regulation of Kir1.1 (ROMK) Renal K⁺ Channels by the Epithelial Sodium Channel*

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The epithelial sodium channel (ENaC) and the secretary potassium channel (Kir1.1/ROMK) are expressed in the apical membrane of renal collecting duct principal cells where they provide the rate-limiting steps for Na⁺ absorption and K⁺ secretion. The cystic fibrosis transmembrane conductance regulator (CFTR) is thought to regulate the function of both ENaC and Kir1.1. We hypothesized that CFTR may provide a regulatory link between ENaC and Kir1.1. In Xenopus laevis oocytes co-expressing both ENaC and CFTR, the CFTR currents were 3-fold larger than those in oocytes expressing CFTR alone due to an increased expression of CFTR in the plasma membrane. ENaC was also able to increase Kir1.1 currents through an increase in surface expression, but only in the presence of CFTR. In the absence of CFTR, co-expression of ENaC was without effect on Kir1.1. ENaC-mediated CFTR-dependent up-regulation of Kir1.1 was reduced with a Liddle’s syndrome mutant of ENaC. Furthermore, ENaC co-expressed with CFTR was without effect on the closely related K⁺ channel, Kir1.4. We conclude that ENaC up-regulates Kir1.1 in a CFTR-dependent manner. CFTR may therefore provide the mechanistic link that mediates the coordinated up-regulation of Kir1.1 during the stimulation of ENaC by hormones such as aldosterone or antidiuretic hormone.

It is becoming clear that membrane transport proteins do not function in isolation but often interact with associated regulatory proteins. An intriguing example of a membrane protein thought to interact with a variety of transport proteins is the cystic fibrosis transmembrane conductance regulator (CFTR). Mutations in CFTR are the underlying cause of cystic fibrosis (CF), a common hereditary disease with pathophysiological abnormalities in a wide range of epithelial tissues. In addition to its role as an epithelial secretory Cl⁻ channel, CFTR has been reported to modify the function of other membrane transport proteins including the amiloride-sensitive epithelial sodium channel (ENaC) and the inwardly rectifying renal outer medullary potassium channel ROMK (Kir1.1). CFTR, ENaC, and Kir1.1 are co-expressed in the apical membrane of principal cells in the renal cortical collecting duct (CCD) where the fine-tuning of renal sodium reabsorption and potassium secretion occurs. We hypothesized that CFTR may be involved in the coordinated regulation of ENaC and Kir1.1.

ENaC provides the rate-limiting step for sodium absorption in a variety of epithelia, particularly in the renal collecting duct. Dynamic regulation of ENaC activity by hormones such as aldosterone and ADH is therefore essential for the maintenance of renal sodium balance and hence for long term regulation of arterial blood pressure. Loss-of-function mutations in ENaC cause urinary sodium loss, hyperkalemia, and low blood pressure in patients with pseudohypoaldosteronism type 1. Conversely, increased ENaC activity in Liddle’s syndrome results in increased sodium re-absorption, hypokalemia, and severe arterial hypertension.

In the lungs of CF patients the failure of defective CFTR to inhibit ENaC is thought to cause hypoabsorption of Na⁺ and fluid possibly contributing to the formation of dry sticky mucus, a hallmark of pulmonary CF pathophysiology. The regulatory relationship between CFTR and ENaC has therefore received considerable attention. Recombinant expression studies (7, 8) have shown ENaC to be inhibited by cAMP-dependent activation of CFTR, and similar observations have been made in various epithelial tissues including mouse renal CCD cells (9). However, the molecular mechanism and physiological relevance of a regulatory relationship between ENaC and CFTR are currently the subject of considerable controversy and may vary in different tissues (8, 10–12).

The complexity of the ENaC-CFTR relationship is further demonstrated by recent co-expression studies (13–15) that suggest that ENaC may have a stimulatory effect on CFTR activity. This ENaC-mediated increase in CFTR currents may be due to altered single channel properties (increased open probability P₀ and/or larger single channel conductance) or to an increase in the overall number of CFTR channels expressed in the membrane. Ji et al. (15) used confocal fluorescence microscopy of oocytes expressing enhanced green fluorescent protein-tagged CFTR to assess CFTR surface expression. They observed an increase in CFTR fluorescence at, or near to, the plasma membrane in the presence of ENaC and suggested that an increase in surface expression may contribute to the ENaC-dependent increase in CFTR activity.

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CFTR is abundantly expressed in the native kidney (16) including the renal collecting duct (9, 17, 18). In immunodetectable CCD cells CFTR was found to be most abundantly expressed in CCD β-intercalated cells but was also detected in α-intercalated cells and in CCD principal cells (18) where it is co-expressed with ENaC (9). It is fair to say that the precise physiological role of CFTR in renal tubular epithelial cells is not yet understood, and renal abnormalities reported in CF patients are subtle (19). A recent study (20) demonstrated that in salt-restricted mice the natriuresis induced by amiloride was significantly greater in CF mice than in wild-type controls, consistent with an increased renal ENaC activity in CF animals. However, an increased renal sodium absorption via ENaC may remain clinically silent in CF patients known to have increased salt losses due to defective salt re-absorption in the ducts of their sweat glands.

One proposed role for CFTR in the kidney is its functional association with Kir1.1 (ROMK) channels to form the native ATP-regulated inwardly rectifying K⁺ channel present in the apical membrane of distal nephron segments, which is responsible for renal potassium secretion. Although heterologously expressed Kir1.1 channels share many characteristics with the native renal secretory K⁺ channel (21), reported differences in their regulation by ATP and the sulfonylurea glibenclamide suggest that Kir1.1 may associate with additional regulatory subunits in vivo. By analogy to the sulfonylurea receptors that confer glibenclamide and nucleotide regulation to the inwardly rectifying K⁺ channels Kir6.1 and Kir6.2, it has been proposed that Kir1.1 may also associate with a renal ABC transporter such as CFTR to form the native secretory K⁺ channel (22, 23).

The precise mechanisms by which CFTR may influence ENaC and Kir1.1 channel activity remain highly controversial. However, a functional interaction between these ion channels may be physiologically important in the renal collecting duct, and CFTR may provide a functional link between ENaC and Kir1.1. To address this question we performed co-expression studies in Xenopus laevis oocytes using two-electrode voltage clamp and patch clamp recordings in combination with an assay to measure surface expression of these ion channels. We found that the observed stimulation of CFTR currents by ENaC can be accounted for by a parallel increase in CFTR surface expression but that ENaC alone has no effect on Kir1.1. However, in the presence of CFTR, ENaC has a large stimulatory effect on Kir1.1 currents by a parallel increase in Kir1.1 surface expression. This functional linkage was largely disrupted by a mutation in ENaC found in Liddle’s syndrome. These findings demonstrate a CFTR-dependent regulation of Kir1.1 by ENaC.

This may provide a physiologically relevant link mediating the concomitant up-regulation of Kir1.1 that is observed during stimulation of ENaC by hormones such as aldosterone and ADH.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—The three subunits of wild-type rat ENaC (rENaC) or of β2ENaC rENaC were in the pS58 vector (gifts from Prof. R. Coster and Prof. L. Schild, Lausanne, Switzerland). Rat Kir1.1 and Kir1.4 were in the oocyte expression vector pBF and human CFTR (a gift from Prof. J.R. Riorian, Mayo Clinic, AZ) in pBluescript KS⁺.

For surface expression studies human CFTR had the hemagglutinin (HA) epitope introduced at amino acid Asn-900 (a gift from Dr. B. Schwappach, Heidelberg, Germany). N-terminal deletion of the first 19 amino acids of Kir1.1a to generate Kir1.1b (ROMK2) (24) was performed by PCR. By using extension overlap PCR the HA epitope (YPYDVPDYA) was introduced into the extracellular loop of Kir1.1a at position 14 together with a glycine residue before and after the epitope. The sequence reads 117GYPYDVPDYAG134. Capped mRNAs were synthesized in vitro by using the T7 or SP6 mMESSAGEmACHINE kit (Ambion, TX).

**Isolation of Oocytes and Injection of cRNA—**X. laevis oocytes were prepared and injected as described (25, 26). Defolliculated oocytes were injected with various cRNA combinations. For each ENaC subunit, or potassium channel, 1 ng of cRNA was used, although 20 ng of cRNA were used for CFTR. 5–10 ng of Kir1.1a-HA cRNA were used to achieve adequate current expression. Injected oocytes were maintained in modified Barth saline (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.3 CaCl₂, 0.4 MgCl₂, 0.82 MgSO₄, 15 HEPES, adjusted to pH 7.6 with Tris) containing 2 μM amiloride to prevent sodium overloading.

**Two-electrode Voltage Clamp Experiments—**Unless stated otherwise, oocytes were studied 2 days after injection using the two-electrode voltage clamp technique as described previously (25, 26). Oocytes were recorded at a holding potential of −60 mV. The barium-sensitive current (ΔIᵦᵦ) was determined by subtracting the corresponding value measured in the presence of 1 mM barium from that measured prior to the application of barium in a KCl solution (in mM: 95 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, adjusted to pH 7.4 with Tris). The amiloride-sensitive current (ΔIᵦᵦ) was determined by subtracting the corresponding current value measured in the presence of 2 μM amiloride from that measured prior to the application of amiloride in a NaCl solution (in mM: 95 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, adjusted to pH 7.4 with Tris). The functional expression of CFTR was verified in each batch of oocytes injected with CFTR cRNA, by demonstrating cAMP-induced activation of Cl⁻ currents upon exposure of the oocytes to IBMX/forskolin (1 mM/1 μM). Data are given as mean values ± S.E.; N indicates the number of oocytes; n indicates the number of batches of oocytes used; significance was evaluated by the appropriate version of Student’s t test.

**Single Channel Patch Clamp Recordings—**Oocytes were assessed by two-electrode voltage clamp recordings to confirm channel expression and were subsequently stripped of the vitellin membrane using sharp forceps and transferred to a bath chamber on a Leica DM IRB inverted microscope (Leitz Microsystems UK Ltd., Milton Keynes, UK). Single channel currents were recorded at room temperature in the “cell-attached” configuration (27) to avoid channel rundown by patch excision. A computer-controlled EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) was used, and experimental procedures were essentially as described previously (28, 29). Patch pipettes were pulled from Clark glass capillaries (Clark Electromedical Instruments, Pangbourne, UK) and were filled with KCl pipette solution (90 mM KCl, 2 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4). They had a resistance of 8.1 megohms (n = 13) in potassium gluconate bath solution (90 mM potassium gluconate, 2 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM HEPES, pH 7.4) which was used in all experiments. In the presence of high extracellular K⁺ the cell membrane of Kir1.1a expressing oocytes is likely to be depolarized, and the trans-patch potential difference is simply defined as the negative pipette holding potential (−Vᵦᵦ), which can be assumed to correspond to the cytoplasmic potential referred to pipette potential. Downward (negative) current deflections correspond to cell membrane inward currents. Current data were filtered at 1 kHz using a sample rate of 5 kHz. Single channel current amplitudes and channel activity (NPᵦ) were estimated from analysis of single channel current amplitudes (29). NPᵦ for each cell was calculated from the single channel current amplitude at −Vᵦᵦ = −120 mV using Ohm’s law. When the apparent number of channels present in a patch recording was greater than 1, NPᵦ was divided by the apparent number of channel levels to give an estimate of single channel open probability (Pₒ), which was determined for each cell-attached patch from a continuous data sample of at least 90 s duration recorded at −Vᵦᵦ = −120 mV. Mean open (tₒ) and closed (t_c) times were obtained from traces containing only one channel. Data were analyzed using the program “Patch for Windows” written by Dr. Bernd Lietz (HEKA Elektronik, Lambrecht/Pfalz, Germany).

**Surface Labeling of Oocytes—**Experiments were essentially performed as described recently (26, 30) using 1 μM [³²P]rat monomolecular anti-HA antibody (clone 3F10, Roche Molecular Biochemicals) as primary antibody and 2 μg/ml peroxidase-conjugated affinity-purified Fab(1/4) fragment goat anti-rat IgG antibody (Jackson ImmunoResearch) as secondary antibody. Chemiluminescence of individual oocytes placed in 50 μl of Power Signal enzyme-linked immunosorbent assay solution (Pierce) was quantified in a Turner TD-20/20 luminometer (Sunnyvale, CA) by integrating the signal over a period of 15 s. Results are given in relative light units.

**Western Blot Analysis—**Oocytes were homogenized using 25 oocytes per experimental group. The homogenate was separated by SDS electrophoresis and transferred to nitrocellulose filters. Primary rat anti-HA monoclonal antibody (100 ng/ml) and secondary peroxidase-conjugated goat anti-rat antibody (160 ng/ml) were diluted in Tris-buffered saline blocking solution. Detection was performed with the enhanced...
[llluminol reagent from PerkinElmer Life Sciences. All Western blot ex-

periments were repeated using two different batches of oocytes.

ENaC co-expression resulted in a significant (**, p < 0.01) increase

in ΔI\text{IBMX-FSK} from an oocyte expressing CFTR alone (Fig. 1C).

Thus, co-expression of ENaC alone does not affect Kir1.1.29% (n = 43;

N = 3; p < 0.001) in ENaC/CFTR-HA oocytes and by 52 ± 12% (n = 39;

N = 3; p < 0.001) in Liddle/CFTR-HA oocytes. These findings therefore demonstrate

that ENaC stimulates CFTR currents by increasing surface expres-

sion of CFTR.

ENaC Alone Has No Effect on Kir1.1—We next examined whether co-expression of ENaC also stimulates Kir1.1. Kir1.1 exists in the kidney in several alternatively spliced isoforms that differ at the distal N terminus. We used the isoform Kir1.1a (ROMK1) known to be expressed in the collecting duct (31). To assess Kir1.1a currents, the Ba2+ (1 mM)-sensitive K+ current (ΔI\text{Ba2+}) was determined in the presence of 95 mM extracellular K+, 48 h after cRNA injection. By contrast to its stimulatory effect on CFTR, co-expression of ENaC did not increase ΔI\text{Ba2+}.

We next examined the mechanism by which ENaC up-regulates CFTR activity, we used an assay that directly measures surface expression of membrane proteins in the plasma membrane of individual oocytes (26, 30). This assay employs chemiluminescent detection of antibody binding to epitopes introduced into the extracellular domains of membrane proteins. We used a version of CFTR with an extracellu-

lar hemagglutinin tag (CFTR-HA), and we measured surface expression in parallel with ΔI\text{IBMX-FSK} from the same group of oocytes. Fig. 1C summarizes the results from one of three similar experiments. ΔI\text{IBMX-FSK} was significantly higher in ENaC/CFTR-HA oocytes compared with that in matched CFTR-HA oocytes. Moreover, the increase in ΔI\text{IBMX-FSK} was paralleled by a similar increase in CFTR-HA surface expression. Consistent with the observations of Ji et al. (15), we found that the stimulatory effect of ENaC on ΔI\text{IBMX-FSK} and surface expression of CFTR-HA was considerably smaller when using ENaC with a Liddle’s syndrome mutation (αβ5γ6γ7 rENaC, Fig. 1C). To rule out an ENaC-dependent increase in CFTR protein synthesis, we performed Western blot analysis on total membrane preparations obtained from the same batch of oocytes. These results demonstrate that the total CFTR-HA protein expression was similar in all three groups of oocytes (Fig. 1C). In three separate experiments ΔI\text{IBMX-FSK} was increased by 173 ± 29% (n = 21; N = 3; p < 0.001) in ENaC/CFTR-HA oocytes and by 47 ± 11% (n = 21; N = 3; p < 0.001) in Liddle/CFTR-HA oocytes when compared with CFTR-HA control oocytes. Similarly, CFTR surface expression was increased by 173 ± 16% (n = 43; N = 3; p < 0.001) in ENaC/CFTR-HA oocytes and by 52 ± 12% (n = 39; N = 3; p < 0.001) in Liddle/CFTR-HA oocytes. These findings therefore demonstrate that ENaC stimulates CFTR currents by increasing surface expression of CFTR.

The Stimulatory Effect of ENaC Is Due to Increased Surface Expression of CFTR—To address the mechanism by which ENaC up-regulates CFTR activity, we used an assay that directly measures surface expression of membrane proteins in the plasma membrane of individual oocytes (26, 30). This assay employs chemiluminescent detection of antibody binding to epitopes introduced into the extracellular domains of membrane proteins. We used a version of CFTR with an extracellular hemagglutinin tag (CFTR-HA), and we measured surface expression in parallel with ΔI\text{IBMX-FSK} from the same group of oocytes. Fig. 1C summarizes the results from one of three similar experiments. ΔI\text{IBMX-FSK} was significantly higher in ENaC/CFTR-HA oocytes compared with that in matched CFTR-HA oocytes. Moreover, the increase in ΔI\text{IBMX-FSK} was paralleled by a similar increase in CFTR-HA surface expression. Consistent with the observations of Ji et al. (15), we found that the stimulatory effect of ENaC on ΔI\text{IBMX-FSK} and surface expression of CFTR-HA was considerably smaller when using ENaC with a Liddle’s syndrome mutation (αβ5γ6γ7 rENaC, Fig. 1C). To rule out an ENaC-dependent increase in CFTR protein synthesis, we performed Western blot analysis on total membrane preparations obtained from the same batch of oocytes. These results demonstrate that the total CFTR-HA protein expression was similar in all three groups of oocytes (Fig. 1C). In three separate experiments ΔI\text{IBMX-FSK} was increased by 173 ± 29% (n = 21; N = 3; p < 0.001) in ENaC/CFTR-HA oocytes and by 47 ± 11% (n = 21; N = 3; p < 0.001) in Liddle/CFTR-HA oocytes when compared with CFTR-HA control oocytes. Similarly, CFTR surface expression was increased by 173 ± 16% (n = 43; N = 3; p < 0.001) in ENaC/CFTR-HA oocytes and by 52 ± 12% (n = 39; N = 3; p < 0.001) in Liddle/CFTR-HA oocytes. These findings therefore demonstrate that ENaC stimulates CFTR currents by increasing surface expression of CFTR.

ENaC Alone Has No Effect on Kir1.1—We next examined whether co-expression of ENaC also stimulates Kir1.1. Kir1.1 exists in the kidney in several alternatively spliced isoforms that differ at the distal N terminus. We used the isoform Kir1.1a (ROMK1) known to be expressed in the collecting duct (31). To assess Kir1.1a currents, the Ba2+ (1 mM)-sensitive K+ current (ΔI\text{Ba2+}) was determined in the presence of 95 mM extracellular K+, 48 h after cRNA injection. By contrast to its stimulatory effect on CFTR, co-expression of ENaC did not increase ΔI\text{Ba2+}.

Stimulation of Kir1.1 by ENaC Is Dependent on the Presence of CFTR—Given the reported regulatory effects of CFTR on both ENaC and Kir1.1, we tested whether the presence of
CFTR influenced the interaction between ENaC on Kir1.1a. We compared the ΔI\textsubscript{Ba\textsuperscript{2+}} in ENaC/CFTR/Kir1.1a oocytes and in matched CFTR/Kir1.1a control oocytes. Average I/V plots are shown in Fig. 2B and demonstrate that ΔI\textsubscript{Ba\textsuperscript{2+}} was significantly increased in ENaC/CFTR/Kir1.1a oocytes coexpressed with CFTR/Kir1.1a oocytes. Individual whole-cell current recordings are shown in Fig. 2, C and D. Note that the Ba\textsuperscript{2+}-sensitive inward current develops slowly after completely substituting Na\textsuperscript{+} by 95 mM K\textsuperscript{+} in the bath solution. This slow activation in high extracellular K\textsuperscript{+} is a well known feature of Kir1.1 currents (24). On average, ΔI\textsubscript{Ba\textsuperscript{2+}} (at -60 mV) was 9.40 ± 0.90 μA (n = 42; N = 6) in ENaC/CFTR/Kir1.1a oocytes and 2.66 ± 0.24 μA (n = 42; N = 6; p < 0.001) in matched CFTR/Kir1.1a controls. We also tested whether ENaC and CFTR can stimulate Kir1.1b (ROMK2), a splice variant of Kir1.1 lacking the first 19 amino acids of the N terminus (31). ΔI\textsubscript{Ba\textsuperscript{2+}} was increased by 99 ± 36% (n = 21; N = 3; p < 0.05) in ENaC/CFTR/Kir1.1b oocytes compared with CFTR/Kir1.1b oocytes. These results demonstrate that ENaC stimulates Kir1.1 currents in a CFTR-dependent manner.

Time Course of Kir1.1 Stimulation by ENaC—The average ΔI\textsubscript{Ba\textsuperscript{2+}} in ENaC/CFTR/Kir1.1a oocytes 18 h after cRNA injection was similar to that in control CFTR/Kir1.1a oocytes. However, a significant stimulatory effect of ENaC was apparent 42 h after cRNA injection and was preserved 66 and 90 h after injection (Fig. 3). The similar currents observed at 18 h rules out the possibility that the stimulatory effect of ENaC was due to a nonspecific effect of the cRNA injection procedure. The relatively late onset of the stimulatory effect of ENaC was probably due to the slower expression of CFTR which is a large ABC protein. The faster expression of Kir1.1a and ENaC currents was in good agreement with previous studies (24, 26) using the oocyte expression system. These results indicate that measurements must be taken at ~48 h after injection to see consistent effects.

ENaC Increases the Surface Expression of Kir1.1a—Given that ENaC stimulates surface expression of CFTR, we examined whether the stimulatory effect of ENaC on ΔI\textsubscript{Ba\textsuperscript{2+}} may be explained by an increased surface expression of Kir1.1a. We therefore measured ΔI\textsubscript{Ba\textsuperscript{2+}} in parallel with surface expression of extracellular HA-tagged Kir1.1a (Kir1.1a-HA). Results from one of two similar experiments are summarized in Fig. 4. These results demonstrate that in ENaC/CFTR/Kir1.1a-HA oocytes,
ΔI_{Ba}^{2+} and surface expression were both increased by about 2.5-fold, compared with the values in CFTR/Kir1.1a-HA control oocytes. On average, ENaC co-expression increased ΔI_{Ba}^{2+} by 128 ± 26% (n = 14; N = 2; p < 0.001) and Kir1.1a-HA surface labeling by 154 ± 26% (n = 19; N = 2; p < 0.001). Interestingly, ENaC with a Liddle’s syndrome mutation (αR564Xγ) had little effect on Kir1.1a-HA currents and surface expression (Fig. 4) consistent with its reduced stimulatory effect on CFTR (Fig. 1C). The Western blot of Kir1.1a-HA shown in Fig. 4 demonstrates that ENaC/CFTR/Kir1.1a-α, CFTR/Kir1.1a-α, and Liddle/CFTR/Kir1.1a-α oocytes express similar levels of Kir1.1a-HA protein.

**ENaC Does Not Alter Single Channel Properties of Kir1.1**—To test the possibility that ENaC co-expression (in the presence of CFTR) alters Kir1.1 channel P_o or single channel conductance, we performed cell-attached patch clamp experiments. As shown in Fig. 5, the single channel activity detected in oocytes expressing ENaC/CFTR/Kir1.1a was similar to that in control oocytes expressing CFTR/Kir1.1a. The observed fast gating kinetics and high P_o values are typical features of Kir1.1 channels (21, 32). In CFTR/Kir1.1a oocytes and in ENaC/CFTR/Kir1.1a oocytes, P_o averaged 0.883 ± 0.004 (n = 8) and 0.883 ± 0.009 (n = 5), respectively. By using the single channel current amplitudes determined at a holding potential of −V_{h} = −120 mV, we estimated an average single channel conductance of 27.3 ± 1.5 pS (n = 8) in CFTR/Kir1.1a oocytes and of 28.6 ± 1.5 pS (n = 5) in ENaC/CFTR/Kir1.1a injected oocytes. By taking into account the potassium concentration of 90 mM in our pipette solution, these single channel conductance values are in good agreement with data reported previously (21, 32). However, in our cell-attached recordings we failed to detect the smaller and variable conductance substrates described previously (23) in inside-out patches from CFTR/Kir1.1a-expressing oocytes. Some patches lasted long enough to determine single channel current amplitudes at various different holding potentials. Data are summarized in Fig. 6 and demonstrate that the single channel current/voltage relationship observed in CFTR/Kir1.1 oocytes was similar to that in ENaC/CFTR/Kir1.1 oocytes. The apparent inward rectification and reversal potentials of 0 mV are consistent with the expected behavior of Kir1.1 channels in symmetrical potassium. The average number of channels per patch observed in ENaC/CFTR/Kir1.1a oocytes was not significantly higher compared with that observed in CFTR/Kir1.1 oocytes averaging 3.0 ± 1.5 (n = 5) and 2.0 ± 0.7 (n = 8), respectively. From recordings with only one visible channel level we analyzed mean open (t_o) and closed (t_c) times. The values obtained in CFTR/Kir1.1a oocytes (t_o = 11.29 ms, t_c = 1.21 ms) were similar to those obtained in ENaC/CFTR/Kir1.1a oocytes (t_o = 12.36 ms; t_c = 1.21 ms) and are in good agreement with the predominant Kir1.1 open time and closed time constants reported previously (32). In conclusion our data do not provide any evidence for an effect of ENaC on Kir1.1a single channel properties. This is in good agreement with our surface expression data that suggest that the ENaC-mediated stimulatory effect on Kir1.1 whole-cell currents is fully accounted for by an increase in the number of Kir1.1 channels expressed at the cell surface.

**ENaC Does Not Affect the Rate of Kir1.1 Retrieval from the Plasma Membrane**—ENaC may increase surface expression of Kir1.1a (in the presence of CFTR) by enhancing its delivery to the cell surface or by inhibiting Kir1.1a retrieval, possibly via competition for the endocytotic machinery. To assess the rate of Kir1.1a retrieval, we inhibited delivery of new channels to the plasma membrane by adding 18 μM brefeldin A (BFA) to oocytes 2 days after injection with cRNA. BFA is a fungal metabolite that inhibits the secretory pathway of newly synthesized proteins without affecting endocytosis (33). Fig. 7 illustrates the effect of BFA on ΔI_{Ba}^{2+} in CFTR/Kir1.1a and ENaC/CFTR/Kir1.1a oocytes. In CFTR/Kir1.1a oocytes, ΔI_{Ba}^{2+} decreased by about 70% within 4 h after addition of BFA (Fig. 7A) which is consistent with recently published data (34) reporting rapid endocytotic retrieval of Kir1.1 from the plasma membrane. In non-treated CFTR/Kir1.1a oocytes ΔI_{Ba}^{2+} continued to increase throughout the 12-h period examined, which suggests that channel insertion exceeded channel retrieval during this period. Importantly, BFA had essentially the same effect on ΔI_{Ba}^{2+} in ENaC/CFTR/Kir1.1a oocytes as in CFTR/Kir1.1a oocytes (Fig. 7B). This demonstrates that co-expression of ENaC

**Fig. 5. Kir1.1a single channel properties are not altered by ENaC.** Cell-attached patch clamp recordings are shown from oocytes expressing CFTR/Kir1.1a (A) or ENaC/CFTR/Kir1.1a (B) using a KCl pipette and a potassium gluconate bath solution (for details see “Experimental Procedures”). Currents were recorded at a holding potential of −V_{h} = −120 mV, and representative continuous current traces are shown. A portion of each current trace indicated by an asterisk is displayed on an expanded time scale to illustrate typical single channel current transitions (note different time bars). Current amplitude histograms were calculated from the complete traces to estimate N_{P_o}. P_o was derived from N_{P_o} assuming that in the traces shown two channels contribute to N_{P_o}.

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**Table 1.** Summary of Single Channel Properties

| Condition | m/s | n | P_o | N_{P_o} |
|-----------|-----|---|-----|--------|
| CFTR/Kir1.1a | 0.883 ± 0.004 | 8 | 1.745 | 1.475 |
| ENaC/CFTR/Kir1.1a | 0.845 | 5 | 1.689 | 1.475 |

*Note: All values are average ± SEM.*
CFTR-dependent Up-regulation of Kir1.1 (ROMK) by ENaC

The main findings of the present study are as follows: 1) co-expression of ENaC increases the cAMP-activated CFTR Cl⁻ currents due to an increase in CFTR surface expression; 2) Kir1.1 currents and surface expression are also increased by ENaC, but only in the presence of CFTR; and 3) the CFTR-dependent interaction between ENaC and Kir1.1 may be defective in Liddle’s syndrome.

It has been reported previously (13–15) that ENaC can increase cAMP-activated CFTR Cl⁻ currents by 2–6-fold when the two channels are co-expressed in Xenopus oocytes. From confocal microscopy with green fluorescent protein-labeled CFTR it was concluded that ENaC enhanced CFTR surface expression (15), whereas single channel analysis suggested that ENaC increased both CFTR Cl⁻ channel open probability and the number of CFTR Cl⁻ channels detected per patch (14).

Our study demonstrates that the increase in cAMP-activated CFTR inward currents by ENaC is predominantly due to an increase in the surface expression of CFTR. However, in the absence of single channel data, we cannot rule out the possibility that an increase in channel open probability may contribute to the effect as suggested in a previous study (14).

The CFTR-dependent stimulatory effect of ENaC on Kir1.1 currents is a novel finding. Kir1.1 potassium currents were not affected by co-expression of ENaC alone. However, in the presence of CFTR, ENaC increased Kir1.1 currents through an increase in surface expression. These findings demonstrate a functional linkage between all three of these channels and suggest that this interaction may provide a mechanism for the coordinate regulation of Na⁺ absorption and K⁺ secretion in the renal collecting duct.

**DISCUSSION**

The Effect of ENaC/CFTR Is Specific to Kir1.1—To test whether other inwardly rectifying K⁺ channels may be affected by co-expression of ENaC, we investigated the effect of ENaC (in the presence of CFTR) on the closely related K⁺ channel Kir1.1 known to be expressed in the basolateral membrane of distal tubular epithelia (35). Fig. 8 illustrates that in ENaC/CFTR/Kir4.1 oocytes, ΔI₁Ba²⁺ was not increased compared with ΔI₁Ba²⁺ in CFTR/Kir4.1 control oocytes. By contrast, in the same batch of oocytes ENaC/CFT stimulated Kir1.1a.

**Mechanism of Up-regulation**—Our data demonstrate that the CFTR-dependent ENaC-mediated increase in Kir1.1 whole-cell currents is due to an increase in Kir1.1 surface expression, whereas Kir1.1 single channel properties were not found to be altered by co-expression of ENaC. The amount of expression of any channel in the plasma membrane is determined by the balance between channel insertion into the membrane and its endocytotic retrieval. Thus ENaC could either increase CFTR and Kir1.1 trafficking and insertion into the plasma membrane or decrease their retrieval. Our BFA experiments indicate that the rate of Kir1.1a retrieval is not affected by ENaC in oocytes co-expression CFTR and Kir1.1a. Hence, the increased Kir1.1a surface expression is most likely due to increased Kir1.1a trafficking to the plasma membrane. Western blots of total membrane fractions showed similar levels of CFTR-HA and Kir1.1a-HA protein expression whether or not ENaC is co-expressed with one or both of these channels. These findings demonstrate that the stimulatory effect of ENaC is not due to an increase in overall channel protein levels by increased biosynthesis or decreased protein degradation. It was also clear that the stimulation of Kir1.1a was not simply due to the hyperactive Liddle’s syndrome ENaC mutant with its increased surface expression (36, 37) had only a modest effect on Kir1.1a compared with wild-type ENaC. Furthermore, the fact that the
Physiological Significance—There is little doubt that hormonal stimulation of renal sodium reabsorption involves both the activation of pre-existing ENaC channels and the insertion of additional ENaC channels in the apical membrane of CCD principal cells. However, the molecular mechanisms and signaling pathways involved in ENaC trafficking are still incompletely understood (3,4). It is also well established that there is obligatory coupling of potassium secretion and sodium reabsorption in the cortical collecting duct (CCD) because increased sodium reabsorption hyperpolarizes the lumen negative trans-epithelial potential difference and thereby enhances the electrical driving force for apical K⁺ exit (47). Indeed, it has been suggested that this increase in driving force may be sufficient to explain the increased K⁺ secretion produced by ADH and mineralocorticoids with no change in the apical membrane K⁺ conductance (48). On the other hand, the potassium conductance of the apical membrane is variable and has been found to be increased by adaptation to high potassium intake, administration of mineralocorticoid hormones, and by ADH (47). Hence, part of the kaliuretic effect of aldosterone and ADH may be attributed to the concomitant stimulation of ENaC and Kir1.1 trafficking to the apical membrane. Our study suggests that ENaC provides the molecular link for a coordinated up-regulation of ENaC and Kir1.1 surface expression. However, we are unaware of observations indicating that patients with cystic fibrosis have an increased incidence of hyperkalemia which suggests that any possible defect in renal collecting duct K⁺ secretion via Kir1.1 is well compensated in these patients. In this context it should be pointed out that in addition to Kir1.1 alternative pathways for K⁺ secretion are believed to be present in the apical membrane of collecting duct cells including a maxi-potassium channel and a K-Cl co-transporter (47).

The observation that α₁β₅γδ ENaC does not up-regulate Kir1.1a suggests that the CFTR-dependent functional linkage between ENaC and Kir1.1a may be defective in Liddle’s syndrome. This is one possible explanation for the clinical observation that in patients with Liddle’s syndrome the degree of renal potassium wasting appears to be rather modest considering the increased driving force for K⁺ secretion in the CCD due to the hyperactive ENaC. Although hypokalemia was emphasized in the original report, this is not a universal finding in patients with Liddle’s syndrome (49) which is compatible with a reduced level of Kir1.1a surface expression in the CCDs of these patients.

The concept that ENaC acts as a regulator of CFTR activity has only started to emerge. This study has, for the first time, directly correlated channel activity with surface expression and demonstrates that ENaC stimulates cAMP-activated CFTR Cl⁻ currents by increasing CFTR surface expression and that CFTR is the mediator in the stimulation of Kir1.1 potassium currents and surface expression by ENaC. These findings suggest that these epithelial ion channels may be functionally coupled in a complex but coordinated fashion to form multi-ion channel units with common physiological regulators.

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