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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 secretory 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, Kir4.1. 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).[1] 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) (1, 2). 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 (3, 4). The analysis of two human genetic diseases has provided direct evidence that molecular dysfunction of ENaC has severe effects on arterial blood pressure. Loss-of-function mutations in ENaC cause urinary sodium loss, hyperkalemia, and low blood pressure in patients with pseudohypoaldosteronism type 1 (5). Conversely, increased ENaC activity in Liddle's syndrome results in increased sodium re-absorption, hypokalemia, and severe arterial hypertension (6).

In the lungs of CF patients the failure of defective CFTR to inhibit ENaC is thought to cause hyperabsorption 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 P_o 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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[1] The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; CCD, cortical collecting duct; ENaC, epithelial sodium channel; rENaC, rat ENaC; ROMK, renal outer medullary potassium channel; HA, hemagglutinin; FSK, forskolin; BFA, brefeldin A; IBMX, isobutylmethylxanthine; ADH, antidiuretic hormone.

CFTR is abundantly expressed in the native kidney (16) including the renal collecting duct (9, 17, 18). In immunodissected 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 β_{R564X} rENaC were in the pSD5 vector (gifts from Prof. B. C. Rossier and Prof. L. Schild, Lausanne, Switzerland). Rat Kir1.1 and Kir4.1 were in the oocyte expression vector pBF and human CFTR (a gift from Prof. J.R. Riordan, 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 113 together with a glycine residue before and after the epitope. The sequence reads ^{113}**Y**GYPYDVPDYAG**P**^{114}. Capped mRNAs were synthesized *in vitro* by using the T7 or SP6 mMESSAGEmMACHINE 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 kept in modified Barth's saline (in mM: 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 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 routinely clamped at a holding potential of −60 mV. The barium-sensitive current ($\Delta I_{Ba^{2+}}$) 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 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, adjusted to pH 7.4 with Tris). The amiloride-sensitive current (ΔI_{ami}) 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 $CaCl_2$, 1 $MgCl_2$, 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 different 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 sharpened 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 $CaCl_2$, 1 mM $MgCl_2$, 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 $MgCl_2$, 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 ($-V_{pip}$), 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 (NP_o) were estimated from amplitude histograms (29). Single channel conductance was calculated from the single channel current amplitude at $-V_{pip}$ = −120 mV using Ohm's law. When the apparent number of channels present in a patch recording was greater than 1, NP_o was divided by the apparent number of channel levels to give an estimate of single channel open probability (P_o), which was determined for each cell-attached patch from a continuous data sample of at least 90 s duration recorded at $-V_{pip}$ = −120 mV. Mean open (t_o) 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 Letz (HEKA Elektronik, Lambrecht/Pfalz, Germany).

Surface Labeling of Oocytes—Experiments were essentially performed as described recently (26, 30) using 1 μg/ml rat monoclonal anti-HA antibody (clone 3F10, Roche Molecular Biochemicals) as primary antibody and 2 μg/ml peroxidase-conjugated affinity-purified $F(ab)_2$ 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

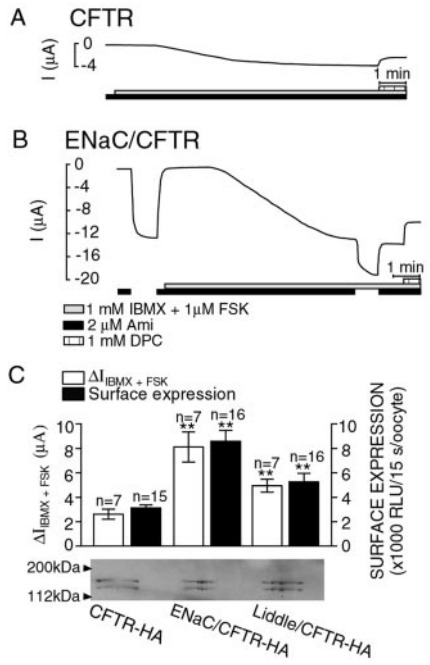

FIG. 1. **Stimulatory effect of co-expression of ENaC on CFTR Cl^- currents and CFTR surface expression.** Representative whole-cell current traces, recorded at −60 mV holding potential, are shown from an oocyte expressing CFTR alone (*A*) and from an oocyte co-expressing ENaC/CFTR (*B*). IBMX (1 mM) and forskolin (*FSK*) (1 μM) were applied to activate cAMP-dependent CFTR Cl^- currents ($\Delta I_{IBMX+FSK}$). Partial inhibition by the chloride channel blocker DPC (1 mM) confirms that $\Delta I_{IBMX+FSK}$ is mediated by CFTR. In ENaC/CFTR oocytes, the amiloride-sensitive whole-cell current (ΔI_{ami}) was evaluated before and after the addition of IBMX-forskolin, confirming functional expression of ENaC and its inhibition by CFTR activation. *C,* surface expression of extracellular hemagglutinin-tagged CFTR (*CFTR-HA*) (*filled bars*) and $\Delta I_{IBMX+FSK}$ (*open bars*) were assessed in parallel in oocytes expressing CFTR-HA alone and in oocytes from the same batch co-expressing either *ENaC/CFTR-HA* or the Liddle's syndrome mutant ($\alpha\beta_{R564X}\gamma$) ENaC with CFTR-HA (*Liddle/CFTR-HA*). ENaC co-expression resulted in a significant (**, $p < 0.01$) increase in $\Delta I_{IBMX+FSK}$ and a concomitant increase in CFTR-HA surface expression expressed in relative light units (*RLU*) per 15 s per oocyte. The stimulatory effect of the Liddle's syndrome mutant was considerably smaller. Western blot analysis of CFTR-HA in total membrane fractions indicates similar levels of expression in all groups. The bands at ~150 and 170 kDa correspond to incompletely glycosylated and mature CFTR-HA, respectively.

luminol reagent from PerkinElmer Life Sciences. All Western blot experiments were repeated using two different batches of oocytes.

RESULTS

Co-expression of ENaC Increases CFTR Cl^- Currents—To understand the mechanism by which ENaC up-regulates CFTR Cl^- currents, we first demonstrated that this effect could be reproduced in our *Xenopus* oocyte expression system. We used a combination of 1 mM IBMX and 1 μM forskolin (IBMX-FSK) to elevate intracellular cAMP and activate whole-cell macroscopic CFTR Cl^- currents that were measured by two-electrode voltage clamp. As shown in Fig. 1*A*, application of IBMX-FSK to CFTR-expressing oocytes caused a sustained stimulation of a characteristic inward current component ($\Delta I_{IBMX+FSK}$, cAMP-activated CFTR chloride current) that was not observed in water-injected control oocytes (data not shown). Moreover, its partial inhibition by the Cl^- channel inhibitor diphenylamine-2-carboxylic acid (DPC) (1 mM) confirmed that $\Delta I_{IBMX+FSK}$ was due to CFTR-mediated Cl^- efflux. Fig. 1*B* shows that $\Delta I_{IBMX+FSK}$ was about 3-fold larger in ENaC/CFTR oocytes (6.86 ± 0.81 μA, $n = 21$, $N = 3$) compared with that in CFTR control oocytes (2.54 ± 0.39 μA, $n = 21$, $N = 3$; $p < 0.001$). Similarly, the DPC-sensitive current component was increased by about 3-fold by co-expression of ENaC. Washout and subsequent re-addition of amiloride (2 μM) prior to the application of IBMX-FSK revealed a sizeable amiloride-sensitive inward current component (ΔI_{ami}) in ENaC/CFTR oocytes confirming functional expression of ENaC (Fig. 1*B*). A second amiloride removal maneuver performed in the presence of IBMX-FSK (Fig. 1*B*) demonstrated that ΔI_{ami} was reduced by 54 ± 4% ($n = 21$; $N = 3$) during cAMP-mediated stimulation of CFTR. This rules out the possibility that cAMP-mediated stimulation of ENaC contributes to the increased $\Delta I_{IBMX+FSK}$ in ENaC/CFTR oocytes and confirms the well established inhibitory effect of cAMP-activated CFTR on ENaC activity. Importantly, our finding that CFTR currents are increased by about 3-fold in ENaC co-expressing oocytes confirm recent studies (13–15) reporting a stimulatory effect of ENaC on 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 $\Delta I_{IBMX+FSK}$ from the same group of oocytes. Fig. 1*C* summarizes the results from one of three similar experiments. $\Delta I_{IBMX+FSK}$ was significantly higher in ENaC/CFTR-HA oocytes compared with that in matched CFTR-HA oocytes. Moreover, the increase in $\Delta I_{IBMX+FSK}$ 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 $\Delta I_{IBMX+FSK}$ and surface expression of CFTR-HA was considerably smaller when using ENaC with a Liddle's syndrome mutation ($\alpha\beta_{R564X}\gamma$ rENaC, Fig. 1*C*). 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. 1*C*). In three separate experiments $\Delta I_{IBMX+FSK}$ was increased by 173 ± 29% ($n = 21$; $N = 3$; $p < 0.001$) in ENaC/CFTR-HA oocytes and by 47 ± 11% ($n = 21$; $N = 3$; $p < 0.01$) 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 Ba^{2+} (1 mM)-sensitive K^+ current ($\Delta I_{Ba^{2+}}$) 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 $\Delta I_{Ba^{2+}}$. Fig. 2*A* shows the results from one of two similar experiments. At a holding potential of −60 mV, $\Delta I_{Ba^{2+}}$ averaged 2.45 ± 0.65 μA ($n = 7$) in ENaC/Kir1.1a oocytes and 2.84 ± 0.15 μA ($n = 7$) in matched Kir1.1a control oocytes (Fig. 2*A*). Thus, co-expression of ENaC alone does not affect Kir1.1a.

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

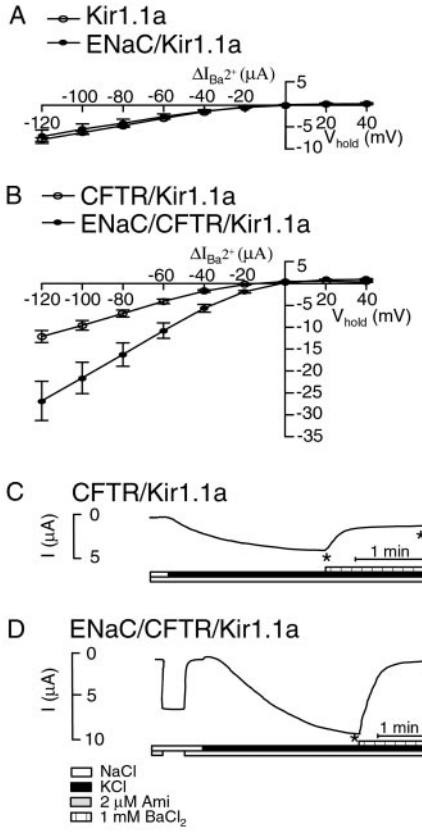

FIG. 2. **Average current/voltage (*I/V*) plots of Ba^{2+} (1 mM)-sensitive K^+ currents ($\Delta I_{Ba^{2+}}$) measured in Kir1.1 expressing oocytes.** From a holding potential of −60 mV, 400-ms voltage steps were performed in 20-mV increments from −120 to +40 mV. $\Delta I_{Ba^{2+}}$ was determined by subtracting the whole-cell currents (using the last 100 ms) recorded in the presence of Ba^{2+} from those recorded prior to its addition. *A*, *I/V* plots from ENaC/Kir1.1a (*filled circles*) and Kir1.1a control oocytes (*open circles*). *B*, *I/V* plots from ENaC/CFTR/Kir1.1a (*filled circles*) and CFTR/Kir1.1a control oocytes (*open circles*). For all groups $n = 7$. Two representative whole-cell current traces, recorded at −60 mV holding potential, are shown from a CFTR/Kir1.1a oocyte (*C*) or from an ENaC/CFTR/Kir1.1a oocyte (*D*). Voltage step protocols were performed at times indicated by *asterisks*, but the resulting current responses were omitted from the continuous trace for clarity.

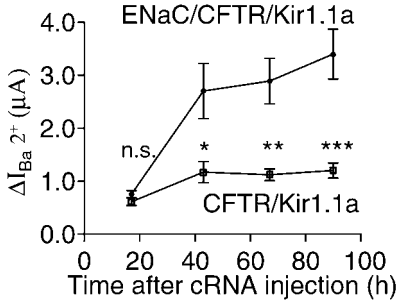

FIG. 3. **Time course of $\Delta I_{Ba^{2+}}$ in ENaC/CFTR/Kir1.1a oocytes (*filled circles*) and matched CFTR/Kir1.1a control oocytes (*open squares*).** At time 0 oocytes from the same batch were injected either with ENaC, CFTR, and Kir1.1a cRNA or with CFTR and Kir1.1a cRNA. ΔIBa^{2+} was assessed in seven oocytes from each group at the times indicated. $\Delta I_{Ba^{2+}}$ was significantly increased in ENaC/CFTR/Kir1.1a oocytes compared with CFTR/Kir1.1a control oocytes for 42 (*, $p < 0.05$), 66 (**, $p < 0.01$), and 90 h (***, $p < 0.001$) after injection. *n.s.*, not significant.

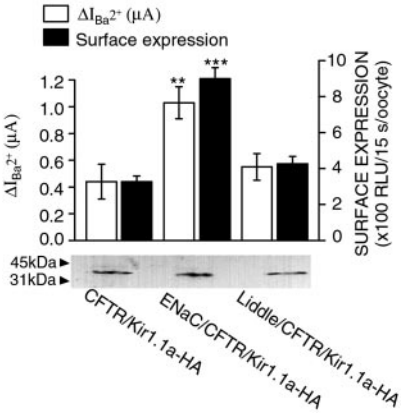

FIG. 4. **ENaC increases the surface expression of Kir1.1a.** Surface expressions of extracellular HA-tagged Kir1.1a (Kir1.1a-HA) and $\Delta I_{Ba^{2+}}$ were assessed in parallel in oocytes co-expressing CFTR/Kir1.1a-HA and in oocytes from the same batch co-expressing either ENaC/CFTR/Kir1.1a-HA or Liddle/CFTR/Kir1.1a-HA. Both surface expression of Kir1.1a-HA (*filled bars*) and $\Delta I_{Ba^{2+}}$ (*open bars*) were significantly increased in ENaC/CFTR/Kir1.1a-HA oocytes compared with CFTR/Kir1.1a-HA control oocytes (**, $p < 0.01$; ***, $p < 0.001$). ENaC with the Liddle's syndrome mutation did not significantly affect $\Delta I_{Ba^{2+}}$ and Kir1.1a-HA surface expression. Seven oocytes per group were used for $\Delta I_{Ba^{2+}}$ measurements and 10 oocytes for detection of surface expression. Western blot analysis of total Kir1.1a-HA protein in oocytes homogenates revealed a band at ~40 kDa and indicated similar expression levels in all groups.

CFTR influenced the interaction between ENaC on Kir1.1a. We compared the $\Delta I_{Ba^{2+}}$ in ENaC/CFTR/Kir1.1a oocytes and in matched CFTR/Kir1.1a control oocytes. Average *I/V* plots are shown in Fig. 2*B* and demonstrate that $\Delta I_{Ba^{2+}}$ was significantly increased in ENaC/CFTR/Kir1.1a oocytes compared with CFTR/Kir1.1a oocytes. Individual whole-cell current recordings are shown in Fig. 2, *C* and *D*. Note that the Ba^{2+}-sensitive inward current develops slowly after completely substituting Na^+ by 95 mM K^+ in the bath solution. This slow activation in high extracellular K^+ is a well known feature of Kir1.1 currents expressed in *Xenopus* oocytes (24). On average, $\Delta I_{Ba^{2+}}$ (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 Kir1.1a lacking the first 19 amino acids of the N terminus (31). $\Delta I_{Ba^{2+}}$ 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 $\Delta I_{Ba^{2+}}$ 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 co-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 Kir1.1—Given that ENaC stimulates surface expression of CFTR, we examined whether the stimulatory effect of ENaC on $\Delta I_{Ba^{2+}}$ may be explained by an increased surface expression of Kir1.1a. We therefore measured $\Delta I_{Ba^{2+}}$ 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,

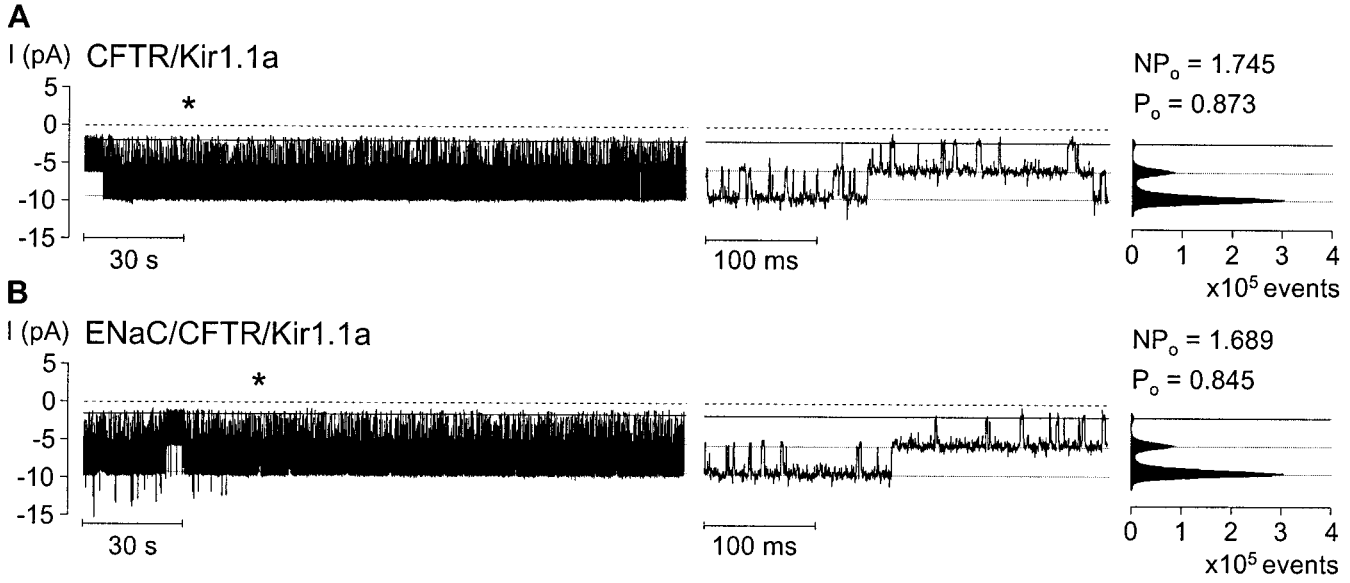

FIG. 5. **Kir1.1a single channel properties are not altered by ENaC.** Cell-attached patch clamp recordings are shown from oocytes expressing CFTR/Kir1.1a (*A*) or ENaC/CFTR/Kir1.1a (*B*) using a KCl pipette and a potassium gluconate bath solution (for details see "Experimental Procedures"). Currents were recorded at a holding potential of $-V_{pip} = -120$ 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 NP_o. P_o was derived from NP_o assuming that in the traces shown two channels contribute to NP_o.

$\Delta I_{Ba^{2+}}$ 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 $\Delta I_{Ba^{2+}}$ 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 ($\alpha\beta_{R564X}\gamma$) had little effect on Kir1.1a-HA currents and surface expression (Fig. 4) consistent with its reduced stimulatory effect on CFTR (Fig. 1*C*). The Western blot of Kir1.1a-HA shown in Fig. 4 demonstrates that ENaC/CFTR/Kir1.1a-HA, CFTR/Kir1.1a-HA, and Liddle/CFTR/Kir1.1a-HA 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 $-V_{pip} = -120$ 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/Kir1.1s 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 substates 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 $\Delta I_{Ba^{2+}}$ in CFTR/Kir1.1a and ENaC/CFTR/Kir1.1a oocytes. In CFTR/Kir1.1a oocytes, $\Delta I_{Ba^{2+}}$ decreased by about 70% within 4 h after addition of BFA (Fig. 7*A*) 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 $\Delta I_{Ba^{2+}}$ 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 $\Delta I_{Ba^{2+}}$ in ENaC/CFTR/Kir1.1a oocytes as in CFTR/Kir1.1a oocytes (Fig. 7*B*). This demonstrates that co-expression of ENaC

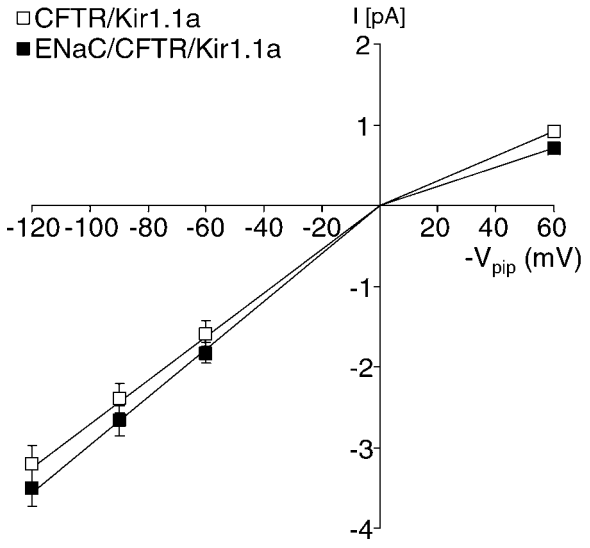

FIG. 6. **The single channel current/voltage (*I/V*) relationship of Kir1.1a is not affected by ENaC.** Average single channel *I/V* plots are shown with each point representing 2–6 single channel current measurements (*vertical bars* indicate S.E. values) from different cell-attached recordings obtained from oocytes expressing CFTR/Kir1.1a (*open squares*) or ENaC/CFTR/Kir1.1a (*filled squares*). Experimental conditions were as described in Fig. 5.

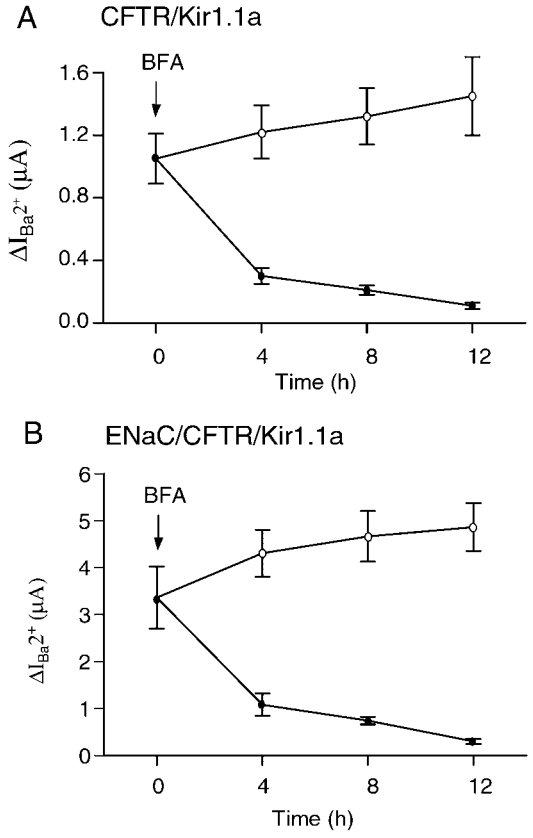

FIG. 7. **Effect of BFA on $\Delta I_{Ba^{2+}}$ in CFTR/Kir1.1a control oocytes (*A*) and in ENaC/CFTR/Kir1.1a oocytes (*B*).** Two days after cRNA injection, oocytes were divided into a control group (*open circles*) and a BFA-treated group (*filled circles*). BFA (18 μM) was added at time 0 as indicated by the ↓, and $\Delta I_{Ba^{2+}}$ was subsequently assessed in 4-h intervals. Each value represents the mean $\Delta I_{Ba^{2+}}$ of seven oocytes. Similar results were obtained in a second batch of oocytes.

does not affect the retrieval rate of Kir1.1a. Hence, the increase of Kir1.1a surface expression by ENaC (in the presence of CFTR) must be due to enhanced delivery of Kir1.1a to the plasma membrane.

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 Kir4.1 known to be expressed in the basolateral membrane of distal tubular epithelia (35). Fig. 8 illustrates that in ENaC/CFTR/Kir4.1 oocytes $\Delta I_{Ba^{2+}}$ was not increased compared with $\Delta I_{Ba^{2+}}$ in CFTR/Kir4.1 control oocytes. By contrast, in the same batch of oocytes ENaC/CFTR stimulated Kir1.1a.

DISCUSSION

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.

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-expressing 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 presence of ENaC in the plasma membrane or to ENaC-mediated sodium loading of the oocytes, because 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

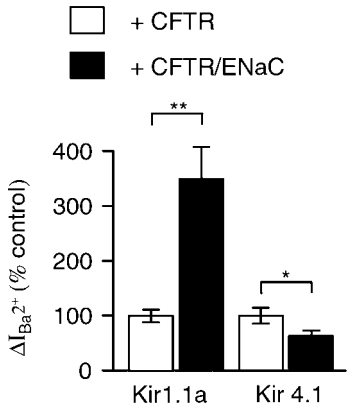

FIG. 8. **ENaC fails to stimulate Kir4.1 in the presence of CFTR.** $\Delta I_{Ba^{2+}}$ was assessed in ENaC/CFTR/Kir1.1a and ENaC/CFTR/Kir4.1 oocytes (*filled bars*) and in CFTR/Kir1.1a- and CFTR/Kir4.1-matched control oocytes from the same batch (*open bars*). To facilitate comparison between Kir1.1a and Kir4.1 currents, the ΔIBa^{2+} values were normalized to the mean $\Delta I_{Ba^{2+}}$ value of the corresponding control group ($n = 14$, $N = 2$ for all groups).

up-regulation of Kir1.1 by ENaC was CFTR-dependent and that the basolateral renal K^+ channel Kir4.1 (35) was not up-regulated in this manner also suggests that this interaction was specific and did not involve a nonspecific effect of ENaC on Kir channel trafficking. The mechanism achieving this linkage therefore is likely to involve either a direct physical interaction between ENaC, CFTR, and Kir1.1 or an indirect interaction through one or more endogenous "adaptor" proteins.

There is some evidence for a direct physical interaction between ENaC and CFTR. Co-immunoprecipitation of *in vitro* translated ENaC and CFTR has been reported (15), and yeast two-hybrid screening has implicated nucleotide-binding fold 1 (NBF1) and the regulatory (R) domain of CFTR in the interaction with ENaC (38). Interestingly, NBF1 and the R domain of CFTR have also been implicated in the regulation of Kir1.1 (39, 40). Therefore, these particular CFTR domains may be involved in the up-regulation of Kir1.1 by ENaC. Whether this involves a direct physical interaction between Kir1.1 and ENaC or CFTR remains to be determined.

An insight into the mechanism involved may come from the observation that the Liddle's syndrome mutation ($\alpha\beta_{R564X}\gamma$) largely reduces the ability of ENaC to up-regulate Kir1.1a in the presence of CFTR. This mutant is a C-terminal deletion of the ENaC β-subunit that deletes the PY motif involved in the regulation of surface expression. This PY motif binds to the WW domain of Nedd4 and facilitates its endocytotic retrieval from the membrane. Deleting this motif therefore increases the amount of ENaC in the plasma membrane (41). Similarly, both Kir1.1 and CFTR have been shown to bind to proteins that regulate intracellular trafficking and localization (1, 2, 42). The bivalent PDZ protein NHERF has been reported to bind to the C terminus of both CFTR and Kir1.1a and may serve to assemble an ion channel complex consisting of Kir1.1 and CFTR (43). A related protein NHERF2 has also recently been shown to be co-localized with Kir1.1 in native renal collecting duct (44), and there are now several examples of adaptor proteins believed to be involved in the assembly and targeting of multiprotein complexes to specific plasma membrane domains in polarized renal tubular epithelial cells (45). It is therefore possible that adaptor proteins containing multiple PDZ domains and WW domains (46) could mediate the interaction and coordinated apical targeting of ENaC, CFTR, and Kir1.1. Interaction with such adaptor proteins could also provide a link to protein kinases or phosphatases to achieve an integrated regulation of these channels.

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 transepithelial 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 CFTR 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 $\alpha\beta_{R564X}\gamma$ rENaC 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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