Vasopressin-stimulated CFTR Cl^- currents are increased in the renal collecting duct cells of a mouse model of Liddle's syndrome

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Liddle's syndrome is a genetic form of hypertension linked to Na^+ retention caused by activating mutations in the COOH terminus of the β or γ subunit of the epithelial sodium channel (ENaC). In this study, we used the short-circuit current (I_{sc}) method to investigate the effects of deamino-8-D-arginine vasopressin (dDAVP) on Na^+ and Cl^- fluxes in primary cultures of cortical collecting ducts (CCDs) microdissected from the kidneys of mice with Liddle's syndrome carrying a stop codon mutation, corresponding to the β-ENaC R_{566} stop mutation (L) found in the original pedigree. Compared to wild-type (+/+) CCD cells, untreated L/+ and L/L CCD cells exhibited 2.7- and 4.2-fold increases, respectively, in amiloride-sensitive (Ams) I_{sc}, reflecting ENaC-dependent Na^+ absorption. Short-term incubation with dDAVP caused a rapid and significant increase (~2-fold) in Ams I_{sc} in +/+, but not in L/+ or L/L CCD cells. In sharp contrast, dDAVP induced a greater increase in 5-nitro-2-(3-phenylpropamino)benzoate (NPPB)-inhibited apical Cl^- currents in amiloride-treated L/L and L/+ cells than in their +/+ counterparts. I_{sc} recordings performed under apical ion substituted conditions revealed that the dDAVP-stimulated apical secretion of Cl^-, which was absent in cultured CCDs lacking CFTR, was 1.8-fold greater in L/+ and 3.7-fold greater in L/L CCD cells than in their +/+ CCD counterparts. After the basal membrane had been permeabilized with nystatin and a basal-to-apical Cl^- gradient had been imposed, dDAVP also stimulated larger Cl^- currents across L/L and L/+ CCD layers than +/+ CCD layers. These findings demonstrate that vasopressin stimulates greater apical CFTR Cl^- conductance in the renal CCD cells of mice with Liddle's syndrome than in wild-type mice. This effect could contribute to the enhanced NaCl reabsorption observed in the distal nephron of patients with Liddle's syndrome.

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Liddle's syndrome is a dominant autosomal form of hypertension (Liddle *et al.* 1963), which results from excessive Na^+ absorption in the distal nephron due to mutations by deletion or modification of a conserved PY motif (PPPxY) in the cytoplasmic COOH termini of β- and γ-ENaC subunits (Shimkets *et al.* 1994; Hansson *et al.* 1995*a*, 1995*b*; Tamura *et al.* 1996).

In the kidney, the fine control of Na^+ absorption takes place in the distal nephron. Na^+ is reabsorbed from the lumen (i.e. from the urine) via the epithelial sodium channel (ENaC), which is composed of four assembled homologous α-, β- and γ-subunits (Canessa *et al.* 1994) located in the apical membrane of the principal distal-collecting duct cells (Duc *et al.* 1994), and is extruded through basolaterally located Na^+,K^+-ATPase (see Garty & Palmer, 1997). Previous heterologous expression studies on *Xenopus* oocytes and cultured cells have demonstrated that the ENaC mutations that cause Liddle's syndrome induce an increase in both the number and activity of ENaC channels and in the retention of the channels at the cell surface (Snyder *et al.* 1995; Schild *et al.*

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1996). These studies have demonstrated the decisive role of the PY motif in the degradation of ENaC at the cell surface via its ubiquitination as a result of binding to the WW domain of the ubiquitin ligase Nedd4–2 (Kamynina *et al.* 2001) and/or endocytosis of the channels via interaction with clathrin-coated pits (Shimkets *et al.* 1997). Pradervand *et al.* (1999*a*) have generated a mouse model of Liddle's syndrome using targeted gene replacement to introduce a R_{566} stop mutation, found in the original pedigree described by Liddle, into the mouse Scnn1b (β-ENaC) gene. Homozygous mutant mice, referred to as L/L mice, replicate many aspects of the human disease (i.e. high blood pressure, metabolic alkalosis, hypokalaemia and low plasma aldosterone levels) when fed a high-Na^+ diet. We showed that intact cortical collecting duct (CCD) cells dissected from the kidneys of these L/L mice exhibit higher transepithelial potential differences, and that in primary cultures they produce greater amiloride-sensitive equivalent short-circuit (I_{eq}) currents than wild-type (+/+) CCD cells (Pradervand *et al.* 2003). This suggests that the Liddle mutation may lead to constitutive hyperactivity of ENaC in the renal CCD. *Ex vivo* and *in vitro* studies have also demonstrated that ENaC activity in CCD cells from L/L mice is still regulated by aldosterone (Auberson *et al.* 2003; Pradervand *et al.* 2003; Dahlmann *et al.* 2003).

Vasopressin and cAMP agonists also stimulate Na^+ absorption in the target cells by increasing the density of ENaC at the cell surface (Marunaka & Eaton, 1991; Morris & Schafer, 2002). The fact that mutations in the PY motif of ENaC can alter the retrieval of the channels at the cell surface (Snyder, 2000, 2002) in transfected rat thyroid cells led to the suggestion that the ENaC mutants responsible for Liddle's syndrome may be less sensitive or even totally insensitive to cAMP agonists. However, other studies of transfected renal CCD cells and dissected CCDs from Liddle syndrome mice (Auberson *et al.* 2003; Dahlmann *et al.* 2003) have demonstrated that ENaC mutants do in fact maintain their ability to respond to vasopressin.

Vasopressin also stimulates cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- conductance in both amphibian A6 cells (Verrey, 1994) and mammalian collecting duct cells (Husted *et al.* 1995; Vandorpe *et al.* 1995; Duong Van Huyen *et al.* 1998; Bens *et al.* 2001; Barriere *et al.* 2003). Co-expression studies in *Xenopus* oocytes, and various cultured cell systems have also shown that CFTR may downregulate ENaC activity (Stutts *et al.* 1995; Ji *et al.* 2000; Konstas *et al.* 2003) and, reciprocally, that CFTR Cl^- conductance can be upregulated by ENaC (Jiang *et al.* 2000). Moreover, the Na^+ current from ENaC mutants carrying Liddle's syndrome mutations can be downregulated by CFTR when coexpressed in *Xenopus* oocytes (Hopf *et al.* 1999). This raises the question as to whether the cAMP-regulated CFTR Cl^- channel is involved in the regulation of NaCl absorption in the renal CCDs of Liddle syndrome mice.

In the present study, we analysed the effects of deamino-8-D-arginine vasopressin (dDAVP), a vasopressin analogue that binds specifically to the V_2 receptor, on ENaC and CFTR activities in renal CCDs from Liddle syndrome mice. Because direct electrophysiological studies of intact CCDs is still technically difficult, we used short-circuit current (I_{sc}) experiments to analyse the electrogenic transport of Na^+ and Cl^- in confluent cultures of single CCD microdissected from the kidneys of wild-type, heterozygous (L/+) and homozygous mutant (L/L) mice. For comparison, we also examined the effects of dDAVP on cultured CCD cells microdissected from the kidneys of m/m mice, in which the Scnn1b gene locus had been partially disrupted (Pradervand *et al.* 1999*b*), and from *cftr*^{m1unc} mice, which lack a functional CFTR (Snouwaert *et al.* 1992).

We demonstrated that short-term (10–20 min) incubation with dDAVP did not produce any further increase in the excessive Na^+ absorption occurring in the renal CCD cells from Liddle syndrome mice, but did hyperstimulate CFTR-dependent Cl^- currents in the apical membrane. This implies that the hyperactivity of the β-ENaC subunit responsible for the Liddle's syndrome phenotype is associated with hyperactive cAMP-stimulated CFTR Cl^- currents. These findings combine to support the idea that the CFTR Cl^- channel could also contribute to the enhanced NaCl absorption that occurs in the distal nephron of Liddle syndrome patients.

Methods

Cell culture

We used heterozygous (L/+) and homozygous (L/L) mutant mice for the Scnn1b allele carrying a premature stop codon corresponding to the R_{566} stop mutation, and their littermate controls, referred to as $+/+^{(L)}$ (Pradervand *et al.* 1999*a*). We also used mice with a homozygous mutant at the Scnn1b locus, referred to as m/m, exhibiting low levels of β-ENaC mRNA expression, and their littermate controls, referred to as $+/+^{(m)}$. Genotyping was carried out by PCR, as previously described (Pradervand *et al.* 1999*a*, 1999*b*). We also used *cftr*^{m1unc} mice, referred to as CFTR (−/−) mice, which lack CFTR-mediated cAMP-dependent Cl^- secretion in the colon, airways, exocrine pancreas (Snouwaert *et al.* 1992) and CCD cells (Bens *et al.* 2001). All these experiments were conducted on 9–13-week-old mice fed a standard diet. The mice were killed by cervical dislocation, the kidneys were rapidly removed under sterile conditions and incubated in a modified defined medium (see below) supplemented with collagenase A (0.1% w/v,

Roche Diagnostics GmBH, Mannnheim, Germany) for 45 min at 37°C. CCDs were then microdissected out under sterile conditions as described (Bens *et al.* 2001). Pools of isolated CCDs (8–12 fragments, 0.2–0.5 mm long) were seeded onto Transwell permeable filters (0.4 μm pore size, 0.33 cm^2 insert growth area, Corning Costar Corp., Cambridge, MA, USA) and grown in a modified defined medium (DM: DMEM: Ham's F12 (1 : 1 v/v), 60 nM sodium selenate, 5 $\mu g\,ml^{-1}$ transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 $ng\,ml^{-1}$ epidermal growth factor (EGF), 5 $\mu g\,ml^{-1}$ insulin, 2% fetal calf serum (FCS), 20 mM Hepes, pH 7.4) at 37°C in a 5% CO_2–95% air atmosphere. After the first five days, the medium was changed every two days. Experiments were carried out two weeks after seeding, using confluent cells that had developed high transepithelial electrical resistance ($>700\ \Omega\,cm^2$). All experiments were performed in accordance with the guidelines of the French Agricultural Office, and in compliance with the legislation governing animal studies.

RNA extraction and RT-PCR

Total RNA was extracted from confluent $+/+^{(L)}$, L/+, L/L and $+/+^{(m)}$ and m/m CCD cells grown on filters using the RNA-PLUS extraction kit (Bioprobe Systems, Montreuil-sous-Bois, France). RNA was reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase (Life Technologies, Eragny, France) at 42°C for 45 min. cDNA and non-reverse-transcribed RNA were amplified for 30 cycles in a total volume of 30 μl containing 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 80 μM dNTP, 1.5 mM $MgCl_2$, 1 unit *Taq* polymerase, 15.7 pmol of α-ENaC, 15.7 pmol of β-ENaC primers or 29 pmol of γ-ENaC primers and 3 pmol of β-actin primers, used as internal standard. The α-ENaC, β-ENaC and γ-ENaC primers used were as previously described (Pradervand *et al.* 2003). RT-PCRs were also performed using 40 pmol of CFTR primers from the mouse CFTR exon 9 and exon 13 and 2 pmol of β-actin primers, as previously described (Bens *et al.* 2001). The thermal cycling program was as follows: 94°C for 30 s, 54°C (for α-ENaC), 56°C (for β-ENaC), 53°C (for γ-ENaC) or 60°C (for CFTR) for 30 s, and 72°C for 60 s. Amplification products were run on 2% agarose gels and photographed.

Quantitative real-time PCR

Total RNA (500 ng) extracted from cultured CCD cells was treated with DNase I (Invitrogen, Cergy Pontoise, France) and reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and 500 ng of random hexamers (Amersham Biosciences Europe, Orsay, France). Reverse-transcribed RNA was subjected to real-time PCR using a ABI7700 sequence detector (Applied Biosystems, Foster City, CA, USA). The primers and Taqman probes for CFTR, α-ENaC and 18S RNA, used as internal control, were as follows: CFTR: upstream primer, 5′-TTGCAGAACAAGACAACACAGTTC-3′, downstream primer, 5′-AAAGAAATCCTTGCACGCTGA-3′, and Taqman probe, 5′-FAM (6-carboxyfluorescin)-TGGAGAAGGTGGAGTCACACTGAGTGGA-TAMRA (6-carboxytetramethylrhodamine)-3′; α-ENaC: upstream primer, 5′-ACCGCATGAAGACGGCC-3′, downstream primer, 5′-CCAGTACATCATGCC-GAAGGT-3′, and Taqman probe: 5′-FAM-CTGGGCG-GTGCTGTGGCTCTG-TAMRA-3′; 18S RNA: upstream primer, 5′-CCCTGCCCTTTGTACACACC-3′, downstream primer, 5′-CGATCCGAGGGCCTCACTA-3′, and Taqman probe: 5′-FAM-CCCGTCGCTACTACCGAT-TGGATGGT-TAMRA-3′. PCR was performed with reverse-transcribed RNA (1/20th of the reverse-transcribed reaction), 3 mM $MgCl_2$ (for 18S) or 5 mM $MgCl_2$ (for CFTR and α-ENaC), 200 μM dNTPs, 1.25 units of *Taq* polymerase, 400 nM of the primers and 100 nM of the corresponding Taqman probes described above. The thermal cycling conditions comprised an initial denaturing step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Standard curves were generated using serial dilutions of an amplified fragment of CFTR (1426–2062 nt), α-ENaC (26–581 nt) and 18S RNA (1428–1788 nt), covering five orders of magnitude and yielding correlation coefficients of at least 0.98 in all experiments. Each standard and samples value was performed in duplicate on RNA samples extracted from four to five separate filters under each of the conditions tested. CFTR and α-ENaC relative mRNA expressions were normalized to 18S RNA, and expressed as the ratio of CFTR over 18S or CFTR over α-ENaC mRNAs.

Electrophysiological studies

CCDs dissected from the kidneys of Scnn1b mutant L/+, L/L and m/m mice and +/+ littermates were seeded and grown on 0.33 cm^2 Transwell filters in DM for 14 days until confluent. They were then equilibrated in DM containing no EGF, hormones, FCS or Hepes, referred to as hormone-free medium or HFM (containing 29 mM $NaHCO_3$), for 2 h before the recordings. Filters were mounted in a modified Ussing-type chamber (diffusion Chamber System, Corning Costar Corp.) connected to a voltage clamp apparatus via glass barrel Micro-Reference Ag/AgCl electrodes. Cell layers were bathed on both sides (0.20 ml for the apical side and 1.2 ml for the basal side) with HFM warmed to 37°C and continuously gassed with 95% O_2–5% CO_2 to maintain a constant pH of 7.4. Cell layers were kept under open-circuit conditions, except when I_{sc} ($\mu A\,cm^{-2}$) was measured by clamping the transepithelial voltage (P_D) to 0 mV for 1 s. By convention,

a positive I_{sc} value corresponded to a flow of positive charges from the apical to the basal compartment. Under these conditions, the transepithelial electrical resistance (R_T) was calculated from P_D and I_{sc} using Ohm's law. For I_{sc} recordings using Cl^--free (ion-substitution experiments) or low Cl^- Ringer solution (basolateral membrane permeabilization experiments), the chambers were first set-up with blank filters to eliminate any voltage offsets and compensate for the resistance of the filters and solutions.

Cl^- and Na^+ substitution experiments

I_{sc} recordings were performed on confluent cultures of CCD cells bathed with Cl^--free or Na^+-free substituted solutions to determine the exact contribution of Na^+ and Cl^- fluxes to the electrogenic transport measured by the short-circuit current method (Duong Van Huyen *et al.* 1998). Experiments were conducted on sets of confluent cells in which the apical and basal $NaHCO_3$−HFM medium was replaced by a Cl^--free solution (mM: 126 sodium gluconate, 1 Na_2HPO_4, 30 $NaHCO_3$, 4.2 $KHCO_3$, 1.05 $CaSO_4$, 20 glucose, 8 Hepes). For comparison, sets of cells were also incubated with the Cl^--free solution added to the apical side of the filter, while the basal side of the filter was bathed with HFM (containing 126 mM Cl^-). Experiments were also conducted on sets of confluent cells in which the apical $NaHCO_3$–HFM medium was replaced by a Na^+-free solution (mM: 156 *N*-methyl-D-glucamine, 4 KCl, 0.7 $MgCl_2$, 0.4 $MgSO_4$, 1.05 $CaCl_2$, 20 glucose, 8 Hepes, pH 7.4). In this case, the basal side of the filter was bathed with HFM continuously gassed with 95% O_2–5% CO_2. All solutions were warmed to 37°C, the basal medium was continuously gassed with 95% O_2–5% CO_2, and I_{sc} recordings were performed after a 40 min equilibration period.

Permeabilization of the basolateral membrane with nystatin

In order to measure the Cl^- currents across the apical membrane, we permeabilized the basolateral membrane of CCD cells using the pore-forming agent nystatin, imposed a Cl^- concentration gradient (basal: 149 mM; apical: 14.9 mM) across the cell layers, and measured I_{sc} by clamping the P_D to 0 mV. We used the protocol described by Li & Sheppard (2003) to measure the apical CFTR Cl^- currents in MDCK cells: under these conditions, cAMP agonists, such as dDAVP, cause the phosphorylation and opening of CFTR Cl^- channels in the apical membrane, thereby generating a transepithelial Cl^- current. On the apical side, confluent cells were bathed in a low Cl^- Ringer solution (mM: 133.3 sodium gluconate, 5 potassium gluconate, 2.5 NaCl, 0.36 K_2HPO_4, 0.44 KH_2PO_4, 5.7 $CaCl_2$, 0.5 $MgCl_2$, 4.2 $NaHCO_3$, 5 glucose, 10 Hepes, pH 7.2 with NaOH), and on the basal side in a Ringer solution (mM: 140 NaCl, 5 KCl, 0.36 K_2HPO_4, 0.44 KH_2PO_4, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 4.2 $NaHCO_3$, 5 glucose, 10 Hepes, pH 7.2 with Tris). After the trace had equilibrated, the basal Ringer solution (1.2 ml) was replaced by warmed Ringer solution supplemented with nystatin (360 μg ml^{-1}). When nystatin was added to the basal side (20 min), the P_D and baseline I_{sc} rapidly reached the 0-value and remained stable for 30 min after the pore-forming nystatin had been added.

Compounds

For all experiments, dDAVP (10^{-8} M, Ferring Pharmaceutical, Malmö, Sweden) was added to the basal side of the filters. Amiloride (Am, Sigma, St Louis, MO, USA), 5-nitro-2-(3-phenylpropamino)benzoate (NPPB; RBI, Natick, MA, USA) were added to the apical side of the filters, while bumetanide (Bum, Sigma) was added to the basal side of the filters. They were dissolved at 0.1 M in DMSO, and diluted 1 : 1000 (10^{-4} M final concentration of NPPB), 1 : 10 000 (10^{-5} M final concentration of Bum). A nystatin solution (360 μg ml^{-1}) was prepared by adding 8.64 μl of the stock solution (50 mg nystatin dissolved in 1 ml DMSO and sonicated for 3 min) to 1.2 ml of warmed basolateral Ringer solution.

Statistics

Results are expressed as means ± S.E.M. Significant difference was analysed by an unpaired Student's *t* test or a one-way ANOVA, using the Bonferroni test or Dunn's test for comparisons of two or more groups. A *P*-value <0.05 was considered significant.

Results

CFTR and ENaC mRNA expression in confluent CCD cells grown on permeable filters

Isolated CCDs microdissected from the kidneys of Scnn1b mutant L/+, L/L and m/m mice and wild-type (+/+) littermates were grown to confluence on Transwell filters. RT-PCR showed that the levels of α-ENaC and γ-ENaC mRNA transcripts were similar in all the types of cultured cells as those of β-actin mRNA transcripts, used as the internal standard (Fig. 1). For β-ENaC, CCD cells from $+/+^{(L)}$ mice exhibited amplified products of the expected size (260 bp), whereas CCD cells from L/+ mice exhibited an additional band (370 bp) due to the simultaneous presence of the mutant Scnn1b L allele (Pradervand *et al.* 1999*a*, 2003) (Fig. 1). The 370-bp band was the only one to be detected solely in the CCD cells from the L/L mice. No amplified products could be detected in cultured CCDs from m/m mice (Fig. 1). No obvious differences in the amounts of CFTR transcripts were apparent (Fig. 1). Quantitative real-time PCR was then performed to determine the amounts

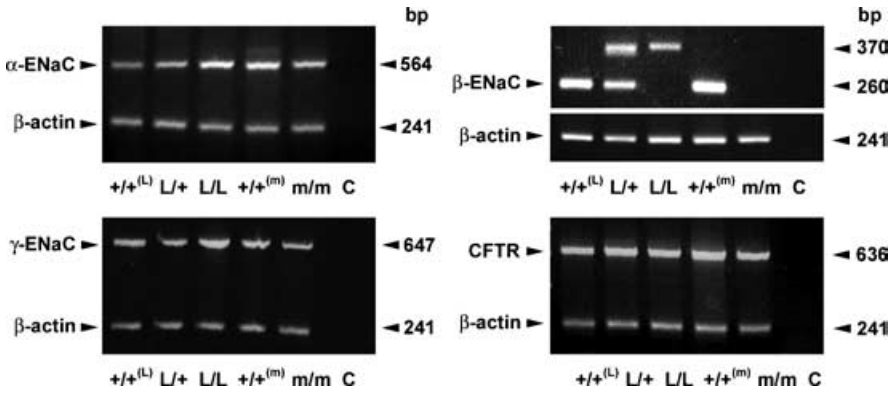

Figure 1. Expression of α-, β- and γ-ENaC and CFTR mRNAs in confluent cultures of CCD cells
cDNA and non-reverse-transcribed RNA from confluent cultures of L/+, L/L, m/m and +/+ CCDs counterparts were amplified by RT-PCR. Each cDNA was amplified (30 cycles) with sets of specific primers for α-, β- and γ-ENaC, CFTR and β-actin, used as internal standard. $+/+^{(L)}$, $+/+^{(m)}$ and L/+ CCD cells yielded one band (260 bp) of β-ENaC amplified products. An additional band (370 bp), corresponding to the L allele, was also detected in cultured L/+ and L/L CCD cells. No amplified β-ENaC expression was detected in m/m CCD cells. As in the controls (C), no amplified products were detected when cDNA was omitted.

of CFTR and α-ENaC mRNA transcripts. All types of cultured CCD cells contained similar amounts of CFTR mRNA relative to the 18S mRNA transcripts, used as the internal standard (Fig. 2). In addition, the ratio of CFTR mRNA over α-ENaC mRNA was similar in Scnn1b mutant and $+/+^{(L)}$ CCD cells (Fig. 2).

Electrophysiological properties

Confluent cultures of CCD cells grown on filters exhibited features typical of a tight epithelium (Table 1). Confluent $+/+^{(L)}$ CCD cells developed high transepithelial electrical resistance ($R_T \sim 1900\ \Omega\ cm^2$) and negative transepithelial voltage ($P_D \sim -30$ mV). L/+ and L/L cells developed significantly lower R_T and significantly greater negative P_D than their $+/+^{(L)}$ cell counterparts (Table 1). It was noteworthy that the P_D values of the L/+ cells were significantly lower than those of the L/L CCD cells (Table 1). I_{sc} values were 2.2-fold and 4.1-fold greater than in the control $+/+^{(L)}$ cells in the L/+ and L/L cells, respectively (Table 1).

As shown in Fig. 3, the addition of amiloride on the apical side induced a similar concentration-dependent decrease in I_{sc} in all cultured Scnn1b mutant CCD cells grown on filters; 10^{-5} M amiloride inhibited the I_{sc} of cultured $+/+^{(L)}$, L/+ and L/L cells by 83–90%. The resulting amiloride-sensitive component of I_{sc} (Ams I_{sc}), which reflects ENaC-mediated Na^+ absorption, was significantly greater than in $+/+^{(L)}$ cells in L/+ and L/L cells, by 2.7-fold and 4.2-fold, respectively (Fig. 3). The amiloride-resistant component of I_{sc} (Amr I_{sc} measured under basal state was also greater in the L/L and L/+ cells (L/L: $9.0 \pm 1.3\ \mu A\ cm^{-2}$, $n = 10$; L/+: 5.7 ± 0.3, $n = 6$) than in the corresponding wild-type CCD cells ($1.4 \pm 1.3\ \mu A\ cm^{-2}$, $n = 8$, $P < 0.001$), but remained low in all cases, i.e. most of the I_{sc} measured under basal condition is due to ENaC-dependent absorption. These findings are in accordance with our previous study (Pradervand *et al.* 2003), and demonstrate that primary cultures of CCDs dissected from the kidneys of L/+ and L/L mutant mice have constitutively increased Na^+ reabsorption capacities.

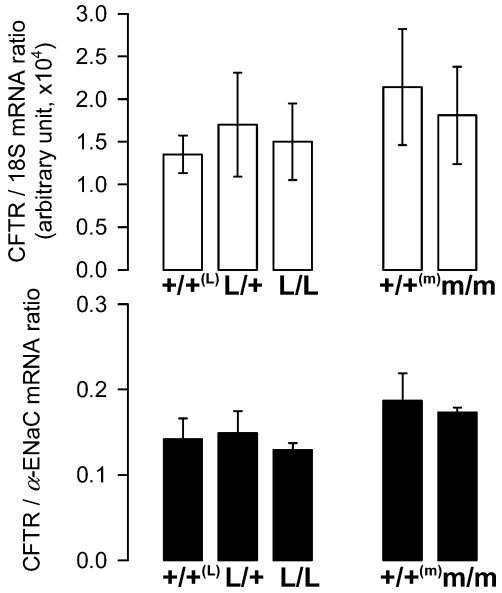

Figure 2. Relative CFTR and α-ENaC mRNA expression in wild-type and Scnn1b mutant CCD cells
The amount of CFTR and α-ENaC mRNAs normalized to 18S RNA were quantified in confluent cultures of L/+, L/L, m/m and +/+ CCDs counterparts by using real-time PCR. Values, expressed as the ratio of CFTR over 18S mRNAs (upper graph) or CFTR over α-ENaC mRNAs (lower graph), are means ± S.E.M. from four to five individual filters measured in each condition tested.

Table 1. Electrophysiological parameters of primary cultured wild-type and Scnn1b mutant CCD cells

CCD	(*n*)	R_T (Ω cm^2)	P_D (mV)	I_{sc} (μA cm^{-2})
+/+$^{(L)}$	(51)	1895 ± 93	− 30.3 ± 1.8	16.6 ± 1.8
L/+	(42)	1488 ± 47^b	− 56.9 ± 1.8^b	39.3 ± 1.0^a
L/L	(41)	1352 ± 44^{b,c}	− 80.4 ± 3.7^{b,d}	69.0 ± 6.7^{b,d}
+/+$^{(m)}$	(30)	1962 ± 100	− 32.4 ± 2.5	17.0 ± 0.6
m/m	(31)	2017 ± 240	− 5.7 ± 0.9^b	3.2 ± 0.4^b

Isolated CCDs were seeded and grown on Transwell filters as described in the Methods section. The transepithelial electrical resistance (R_T), voltage (P_D), and short-circuit current (I_{sc}) were measured under basal conditions in confluent CCD cells dissected from L/+, L/L and m/m mice and the corresponding wild-type +/+$^{(L)}$ and +/+$^{(m)}$ littermates. Values are means ± S.E.M. from (*n*) individual measurements. $^a P < 0.01$, $^b P < 0.001$ *versus* wild-type +/+ values; $^c P < 0.05$, $^d P < 0.01$ *versus* L/+ values.

CCD cells from m/m mutant mice developed R_T values comparable to those of their wild-type CCD counterparts, but exhibited a less negative P_D (Table 1). I_{sc} values from m/m cells were very small, and corresponded to only 23% and 6% of the Ams I_{sc} found in +/+$^{(m)}$ and L/L CCD cells, respectively (Fig. 3). The Amr I_{sc} values were quite similar in both +/+$^{(m)}$ (1.8 ± 0.7 μA cm^{-2}, $n = 7$) and m/m cells (1.4 ± 0.5 μA cm^{-2}, $n = 5$).

Contrasting effects of deamino-8-D-arginine vasopressin in wild-type and Scnn1b mutant CCD cells

We next investigated the short-term effects of dDAVP, an analogue of vasopressin that binds specifically to the V_2 receptors, on confluent cultures of CCD cells. All experiments were performed using 10^{-8} M dDAVP, a concentration that had been shown to induce maximal Na^+ absorption in cultured mouse CCD cells (Duong Van Huyen *et al.* 1998). We first analysed the effects of the basal addition of 10^{-8} M dDAVP for 20 min on P_D and R_T that were measured under open-circuit conditions before clamping P_D to 0 mV for I_{sc} measurements (Table 2). dDAVP caused a significantly greater decrease in R_T in L/+ (−50%) and L/L (−58%) cells than in +/+$^{(L)}$ cells. In addition, dDAVP caused a dramatic shift of P_D to positive values in L/+ and L/L cells that was not observed in the control +/+$^{(L)}$ cells (Table 2).

Under short-circuit conditions, dDAVP induced a progressive rise in I_{sc} that plateaued 10 min after adding the polypeptidic hormone to +/+ cells. dDAVP also caused a more rapid rise in I_{sc} in both L/+ and L/L cells (Fig. 4). As shown in Table 2, the magnitude of the increase in total I_{sc} (ΔI_{sc}) caused by dDAVP was very similar in +/+$^{(L)}$, L/+ and L/L cells (ΔI_{sc}; +/+$^{(L)}$ cells: 16.5 ± 1.9 μA cm^{-2}, $n = 8$; L/+ cells: 12.2 ± 1.9 μA cm^{-2}, $n = 7$, L/L cells: 15.2 ± 3.6 μA cm^{-2}, $n = 9$) but very small ($P < 0.01$) in m/m cells (ΔI_{sc}: 3.5 ± 0.5 μA cm^{-2}, $n = 6$). These results indicate that both L/+ and L/L and +/+ CCD cells have comparable sensitivity towards AVP. However, the significant increase in Ams I_{sc} caused by dDAVP in +/+$^{(L)}$ cells was not observed in L/+ and L/L CCD cells (Fig. 4). It is therefore suggested that, in addition to ENaC, dDAVP may stimulate other types of conductance in CCD cells carrying the activating Scnn1b L mutation.

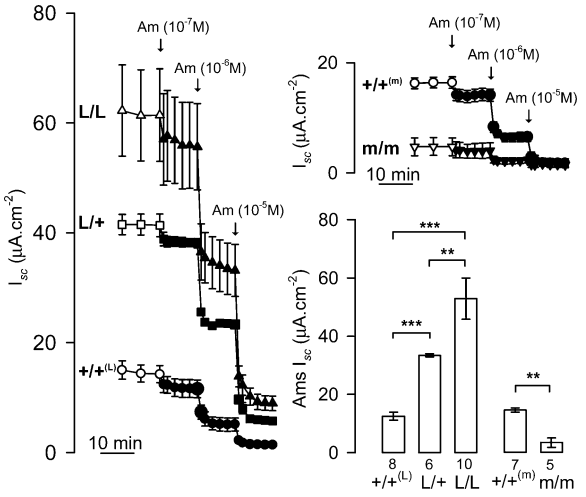

Figure 3. I_{sc} in untreated cultures of CCD cells
I_{sc} was measured in sets of confluent cultures of L/+, L/L, m/m and +/+ CCDs counterparts grown on filters before (open symbols) and after sequential apical additions of 10^{-7} M, 10^{-6} M and 10^{-5} M amiloride (filled symbols). The bars represent Ams I_{sc} values measured after adding 10^{-5} M amiloride. Values are means ± S.E.M. from five to ten separate filters in each condition tested. $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* +/+ values.

Deamino-8-D-arginine vasopressin stimulates Cl^- secretion under short-circuit conditions

dDAVP significantly increased the Amr component of I_{sc} in +/+$^{(L)}$ cells (−dDAVP: 1.2 ± 0.2 μA cm^{-2}; +dDAVP: 6.8 ± 1.1 μA cm^{-2}, $n = 8$, $P < 0.001$), L/+ cells (−dDAVP: 6.3 ± 0.3 μA cm^{-2}; +dDAVP: 10.3 ± 1.2 μA cm^{-2}, $n = 7$, $P < 0.01$) and L/L cells (−dDAVP: 9.3 ± 1.6 μA cm^{-2}; +dDAVP: 26.5 ± 2.4 μA cm^{-2}, $n = 9$, $P < 0.001$) (see Fig. 4). Vasopressin stimulates major Cl^- reabsorption under physiological open-circuit conditions in amphibian A6 cells (Verrey, 1994), and in rat or mouse collecting duct cells, and stimulates CFTR-mediated Cl^- secretion under short-circuit conditions (i.e. I_{sc} condition) (Husted *et al.* 1995; Vandorpe *et al.* 1995; Bens *et al.* 2001). The increase in Amr I_{sc} caused by dDAVP in the three types of cells cultured may well correspond to a basal-to-apical flow of negative charges (i.e. Cl^-). This point was checked in experiments on wild-type cells incubated with a Na^+-rich, Cl^--free solution on both the apical and basal sides. The rise in total I_{sc} caused by dDAVP was lower than that observed in the presence of apical and basal

Table 2. Effects of dDAVP on electrophysiological parameters in primary cultured wild-type and Scnn1b mutant CCD cells

CCD	(*n*)	R_T (Ω cm^2)		P_D (mV)		I_{sc} (μA cm^{-2})	
		−dDAVP	+dDAVP	−dDAVP	+dDAVP	−dDAVP	+dDAVP
$+/+^{(L)}$	(8)	1854 ± 186	1297 ± 79^a	-28.9 ± 6.2	-32.0 ± 4.5	17.31 ± 1.8	33.8 ± 3.6^b
L/+	(7)	1563 ± 108	781 ± 59^c	-57.0 ± 1.6	-36.8 ± 2.3^c	35.4 ± 2.3	47.6 ± 2.6^a
L/L	(9)	1353 ± 83	571 ± 26^c	-87.2 ± 6.8	-45.1 ± 2.8^b	65.3 ± 1.8	80.5 ± 3.9^a
m/m	(6)	1785 ± 75	1444 ± 22^a	-2.7 ± 0.8	-7.5 ± 2.1	1.6 ± 0.2	5.2 ± 0.6^c

The transepithelial electrical resistance (R_T), voltage (P_D), and short-circuit current (I_{sc}) were measured in confluent cultures of CCD cells from $+/+^{(L)}$ L/+, L/L and m/m CCD cells grown on Transwell filters before (−dDAVP) and 20 min after the basal addition of 10^{-8} M dDAVP (+dDAVP). Values are means ± S.E.M. from (*n*) individual measurements.[a] $P < 0.05$,[b] $P < 0.01$,[c] $P < 0.001$ *versus* −dDAVP values.

HFM in $+/+^{(L)}$ cells (see Figs 4 and 5). It increased significantly when the Cl^--free solution bathing the basal side of the filters was replaced by a 126 mM Cl^--enriched solution (HFM) (Fig. 5, upper panels). Under these latter incubation conditions, the $\triangle I_{sc}$ increase in total I_{sc} in response to dDAVP challenge was mainly due to a greater Amr I_{sc}. There was no change in the Ams I_{sc} component when compared to the same set of $+/+^{(L)}$ cells bathed with the Na^+-rich, Cl^--free solution on both apical and basal sides (Fig. 5, upper right-hand panel). Therefore, in $+/+^{(L)}$ cells, part of the increase in I_{sc} caused by dDAVP stimulation is due to the secretion of Cl^-.

dDAVP had no effect on the total I_{sc} when L/L CCD cells were bathed with a Na^+-rich, Cl^--free solution on both apical and basal sides (Fig. 5, lower panels). However, when the basal Cl^--free solution was replaced by HFM containing 126 mM Cl^-, it did induce a significant rise in I_{sc} and Amr I_{sc}, but had no effect on Ams I_{sc} as compared to the same set of L/L cells bathed with the Cl^--free solution on both apical and basal sides (Fig. 5, lower right-hand panel). Altogether, these results indicated that under short-circuit conditions, the rise in total I_{sc} induced by dDAVP corresponds to the activation of apical Cl^- curents in $+/+^{(L)}$ and L/L CCD cells.

dDAVP hyperstimulates Cl^- secretion in L/L CCD cells

Apical addition of 10^{-5} M amiloride, which almost completely inhibited ENaC activity (see Fig. 3), caused a rapid fall in I_{sc} in $+/+^{(L)}$, L/L and m/m cells incubated in HFM (Fig. 6). The I_{sc} increase produced when dDAVP was subsequently added was significantly greater in L/L cells than in $+/+^{(L)}$ cells (Fig. 6). The disruption of Scnn1b in m/m cells did not significantly affect the slight rise in I_{sc} caused by dDAVP (Fig. 6). In all cases, the subsequent apical addition of 10^{-4} M NPPB, a potent Cl^- channel blocker in the mouse CFTR (Bens *et al.* 2001), reduced the dDAVP-stimulated I_{sc} by 25% to 45% (Fig. 6). Thus, the blockade of ENaC unmasked a component of I_{sc} stimulated by dDAVP that was partially inhibited by NPPB, presumably corresponding to a fraction of secreted Cl^- mediated by CFTR (Bens *et al.* 2001).

The dDAVP-stimulated apical Cl^- conductance is increased in L/L CCD cells

The question arose as to whether the increase in dDAVP-stimulated Cl^- secretion in L/+ and L/L CCD cells reflected an increase in apical Cl^- conductance and/or was the consequence of an increase in the Na^+ transport rate, and possibly more active basolateral Na^+,K^+-ATPase and basolateral K^+ channel(s), which could account for a more negative membrane potential, and hence a greater driving force for the exit of Cl^-. To answer this question, the electrical properties of the basolateral membrane were neutralized by permeabilizing the basolateral membrane with nystatin. Nystatin has the advantage of creating aqueous pores that are large enough to allow monovalent ions and water to permeate, and thus avoid any manipulations involving Ca^{2+} or ATP, since divalent cations and ATP are both too large to pass through nystatin pores (Rae & Fernandez, 1991; Li & Sheppard, 2003). The I_{sc} recordings were then performed under a Cl^- basal (149 mM) to apical (14.9 mM) gradient. I_{sc} and P_D reached zero-values 20–30 min after the basal addition of nystatin (Fig. 7). To make sure that the basolateral membrane had been permeabilized, we checked that a subsequent basal addition of ouabain did not modify the currents recorded in L/L cells (nystatin: 0.2 ± 0.9 μA cm^{-2}; nystatin plus ouabain: -0.4 ± 0.2 μA cm^{-2}, $n = 4$) and $+/+^{(L)}$ CCD cells (data not shown). In both $+/+^{(L)}$ and L/L cells, the basal addition of dDAVP induced a rapid rise in I_{sc} which remained stable for 10 min (and for longer times, not shown). As shown in Fig. 7, the magnitude of the increase in I_{sc} ($\triangle I_{sc}$) caused by dDAVP was significantly 1.9-fold greater in L/L CCD cells than in their $+/+^{(L)}$ cell counterparts ($\triangle I_{sc}$ $+/+^{(L)}$ cells: 22.1 ± 2.2 μA cm^{-2}, $n = 6$; L/L cells: 45.3 ± 3.8 μA cm^{-2}, $n = 7$, $P < 0.001$). The increase in I_{sc} induced by dDAVP was also significantly greater in L/+ CCD cells ($\triangle I_{sc}$; L/+ cells: 38.2 ± 4.2 μA cm^{-2}, $n = 5$, $P < 0.01$) than in wild-type cells (Fig. 7). The

increase in I_{sc} elicited by dDAVP must have corresponded to the Cl^- current, since it was totally inhibited by the subsequent apical addition of 10^{-4} M NPPB (Fig. 7). Moreover, the subsequent apical addition of amiloride did not alter the NPPB-inhibitable currents (data not shown). Thus, these experimental data indicate that the dDAVP-stimulated Cl^- conductance measured after basolateral membrane permeabilization is significantly greater in CCD from mice carrying the activating Scnn1b L mutation.

Then the question arises as to whether the observed increase in dDAVP-stimulated Cl^- conductance is specific for ENaC with the Liddle's syndrome mutation, or whether any increase in ENaC-mediated Na^+ absorption would also result in an increase in Cl^- secretion. I_{sc} recordings were therefore undertaken on $+/+^{(L)}$ CCD cells in which Na^+ absorption was stimulated by aldosterone (Aldo). Incubating $+/+^{(L)}$ cells for 4 h with 10^{-6} M Aldo significantly increased total I_{sc} as compared to untreated cells (control: 26.1 ± 2.4; Aldo: $43.7 \pm 3.2\ \mu A\ cm^{-2}$, $n = 7$, $P < 0.001$). Under the conditions of basolateral membrane permeabilization with nystatin and an imposed Cl^- gradient, the increase in ΔI_{sc} elicited by dDAVP was significantly greater in aldosterone-treated wild-type $+/+^{(L)}$ CCD cells ($37.6 \pm 6.2\ \mu A\ cm^{-2}$, $n = 7$, $P < 0.05$) than in untreated $+/+^{(L)}$ CCD cells (Fig. 7). These findings suggest that the magnitude of the Cl^- secretory response to dDAVP depends to a large extent on the magnitude of the Na^+ absorption mediated by ENaC.

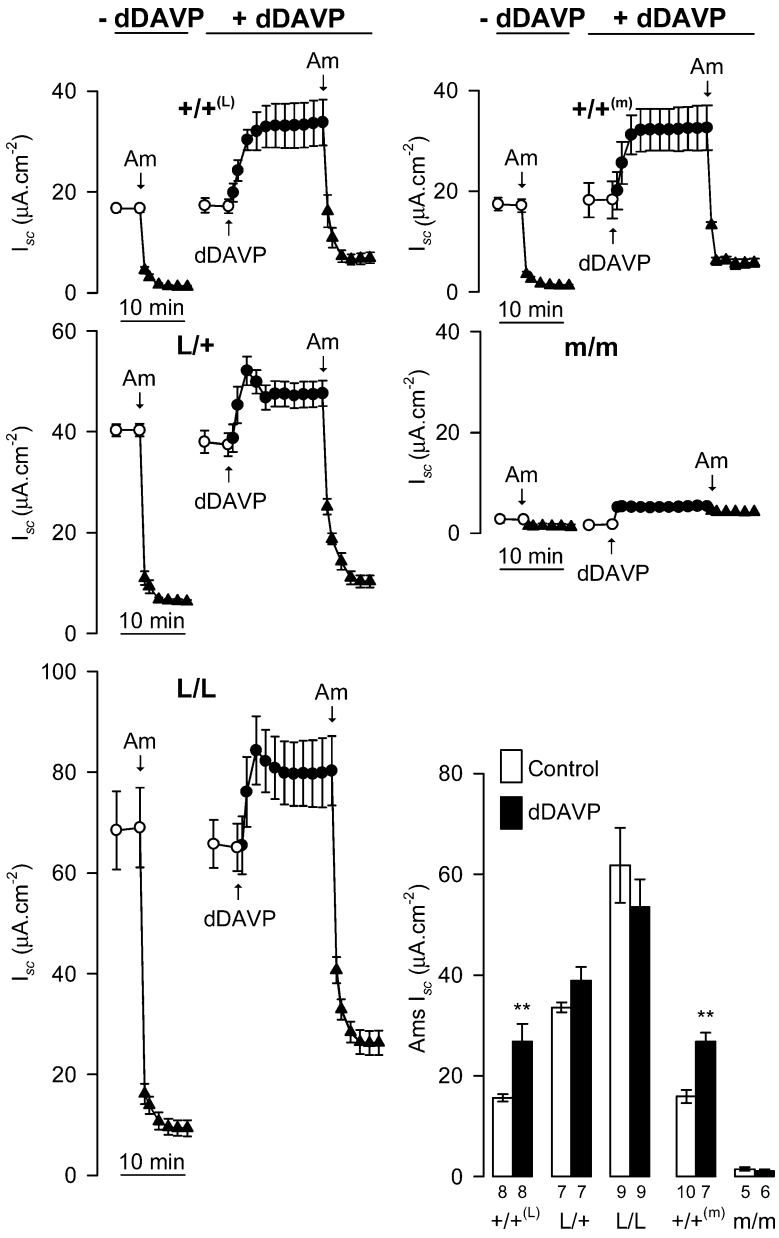

Figure 4. Effects of dDAVP on I_{sc} on confluent cultures of wild-type and Scnn1b mutant CCD cells

I_{sc} was measured in sets of confluent $+/+^{(L)}$, L/+, L/L, $+/+^{(m)}$ and m/m CCD cells grown on filters before (○) and after adding amiloride (10^{-5} M, ▲) or sequential additions of 10^{-8} M dDAVP (●) and amiloride (▲). Amiloride was added to the apical side of the filters, whereas the dDAVP was added to the basal side of the filters. The bars represent Ams I_{sc} values from untreated (open bars) and dDAVP-treated cells (filled bars) after adding 10^{-5} M amiloride. Values are means ± S.E.M. from five to ten separate filters in each condition tested. $^{**}P < 0.01$ *versus* untreated +/+ values.

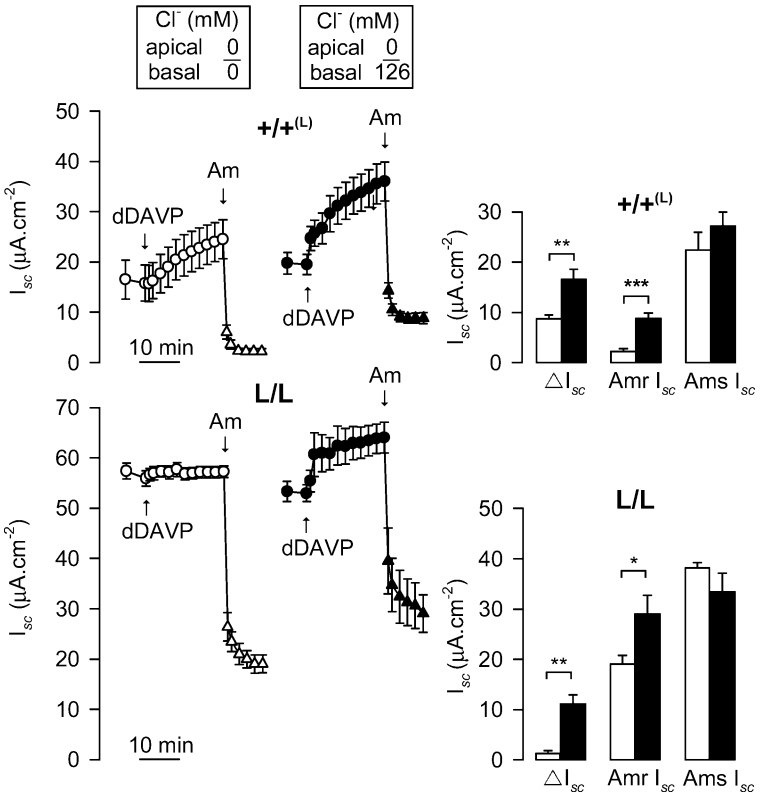

Figure 5. Effects of apical and basal substitution of Cl^- on the I_{sc} elicited by dDAVP in wild-type and Scnn1b mutant L/L CCD cells
Sets of confluent +/+$^{(L)}$ (upper graphs) and L/L (lower graphs) CCD cells were incubated with apically and basally Cl^- substituted (Cl^-, 0 mM) solutions (○) or with apical Cl^--substituted solution and basal HFM (Cl^-, 126 mM) (●) as described in Methods. After equilibrating the traces, I_{sc} was measured before and after sequential additions of basal dDAVP (10^{-8} M) and apical amiloride (10^{-5} M). ΔI_{sc} represents the relative increase in I_{sc} induced by dDAVP. Amr I_{sc} and Ams I_{sc} represent the amiloride-resistant and -sensitive components, respectively, of I_{sc} measured in cells incubated with apical and basal Cl^--substituted solutions (open bars) or apical Cl^--substituted solution and basal HFM (filled bars). Values are means ± S.E.M. from five separate experiments for each condition tested. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between groups.

The Cl^- secretion elicited by deamino-8-D-arginine vasopressin is mediated by CFTR

We have previously shown that CCD cells incubated on their apical side with a Na^+-free solution (in which Na^+ was replaced by *N*-methyl-D-glucamine) plus amiloride (10^{-5} M) and with HFM on their basal side, made it possible to obtain reproducible I_{sc} recordings in which any participation of apical ENaC-dependent Na^+-absorption was excluded (Bens *et al.* 2001). It was impossible to exclude the possibility that some Na^+ remained in the apical compartment after it had been washed and the apical (200 μl) HFM medium replaced by the Na^+-free medium, and so amiloride was always added to the apical Na^+-free solution to ensure that there was no participation of ENaC. The I_{sc} was measured under these conditions to define quantitatively the dDAVP-dependent secretion of Cl^- in the Scnn1b mutant L/L and m/m CCD cells and +/+ counterparts (Fig. 8). Adding dDAVP to the basal side of the cells caused a rapid (1 min), significant rise in I_{sc} in all cultured CCD cells that was inhibited (~40%) by the subsequent addition of apical NPPB (Fig. 8). These findings provided direct evidence that dDAVP stimulated the secretion of Cl^- into the apical medium under short-circuit current conditions. We have previously shown that under open-circuit conditions, dDAVP stimulated both the predominant Cl^- absorption and a minor component of Cl^- secretion in primary cultures of wild-type CCD cells (Bens *et al.* 2001). The

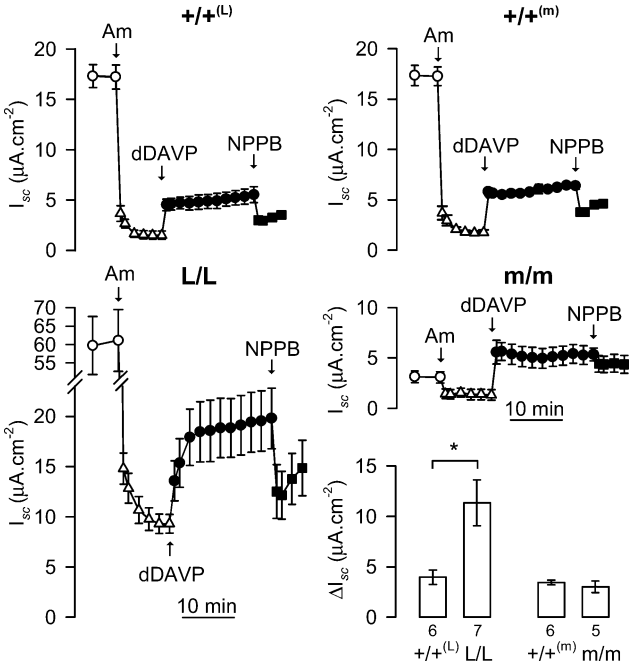

Figure 6. Effects of amiloride and NPPB on dDAVP-stimulated I_{sc}
I_{sc} was measured in sets of confluent cultures of +/+$^{(L)}$, L/L, +/+$^{(m)}$ and m/m CCD cells grown on filters and sequentially incubated without (○) or with apical amiloride (Am, 10^{-5} M, Δ), basal dDAVP (10^{-8} M, ●) and apical NPPB (10^{-4} M, ■). Bars represent the relative ΔI_{sc} increase caused by dDAVP. Values are means ± S.E.M. from five to seven separate experiments for each condition tested. $^*P < 0.05$ between groups.

stimulation of basal-to-apical transcellular flux of Cl^- was confirmed by the fact that the basal addition of 5×10^{-5} M bumetanide (Bum) for 10 min, used to block the electroneutral Na^+,K^+,$2Cl^-$ cotransporter, prior to the basal addition of dDAVP impaired, at least partially, the increase in ΔI_{sc} caused by dDAVP in both $+/+^{(L)}$ (−Bum: $2.5 \pm 1.0\ \mu A\ cm^{-2}$, $n = 8$; +Bum: $0.9 \pm 1.0\ \mu A\ cm^{-2}$, $n = 4$) and L/L cells (−Bum: $9.6 \pm 0.3\ \mu A\ cm^{-2}$, $n = 6$; + Bum: $1.9 \pm 0.1\ \mu A\ cm^{-2}$, $n = 3$).

In sharp contrast to what we observed in wild-type and Scnn1b L/+ and L/L mutant CCD cells, in CFTR −/− CCD cells, neither dDAVP nor NPPB had any effect on I_{sc} (Fig. 8). These findings confirmed our previous observation that the increase in Cl^- secretion caused by dDAVP is mediated by CFTR (Bens *et al.* 2001). Our data also show that the increase in ΔI_{sc} caused by dDAVP is significantly greater in L/+ and L/L cells than in $+/+^{(L)}$ cells. The progressive increase in the dDAVP-dependent ΔI_{sc} observed between $+/+^{(L)}$ cells ($+ 2.5 \pm 0.3\ \mu A\ cm^{-2}$, $n = 7$), L/+ cells ($+ 4.7 \pm 0.3\ \mu A\ cm^{-2}$, $n = 8$) and L/L cells ($+ 9.6 \pm 0.5\ \mu A\ cm^{-2}$, $n = 6$) demonstrates that the *in vivo* expression of an activating Scnn1b mutation (L/+, L/L) in transgenic mice is closely associated with an increase in cAMP-dependent CFTR activity in renal CCD cells.

NPPB blunted the secretion of Cl^- elicited by dDAVP, and so we carried out tests to find out whether this Cl^- channel blocker altered Na^+ absorption in wild-type and/or L/L CCD cells incubated with HFM on both sides of the filters. Apical addition of NPPB (10^{-4} M for 10 min) followed by the basal addition of dDAVP did not significantly alter Ams I_{sc} in L/L cells (−NPPB: $53.4 \pm 5.5\ \mu A\ cm^{-2}$, $n = 9$; + NPPB: $61.0 \pm 13.0\ \mu A\ cm^{-2}$, $n = 5$) and $+/+^{(L)}$ cells (−NPPB: $26.9 \pm 3.4\ \mu A\ cm^{-2}$, $n = 8$; +NPPB: $17.8 \pm 7.8\ \mu A\ cm^{-2}$, $n = 6$).

Isoproterenol acting on intercalated cells has similar effects on Cl^- secretion in wild-type, L/L and m/m CCD cells

The renal collecting duct is composed of two main cell types: principal and intercalated cells, and both cell types express CFTR (Todd-Turla *et al.* 1996). Additional experiments were therefore performed using isoproterenol (ISO), a β-adrenergic agonist known to produce specific stimulation of cAMP in intercalated cells (Fejes-Tóth & Náray-Fejes-Tóth, 1989). The increase in total I_{sc} caused by the basal addition of 10^{-5} M ISO ($\Delta I_{sc} \sim +9\ \mu A\ cm^{-2}$) was almost identical in $+/+^{(L)}$, L/L and m/m cells (Fig. 9). Moreover, the levels of Ams I_{sc} remained unchanged in untreated and ISO-treated L/L, m/m cells and $+/+^{(L)}$ cells (Fig. 9). These findings indicate that the cAMP-dependent Cl^- secretion that occurs in

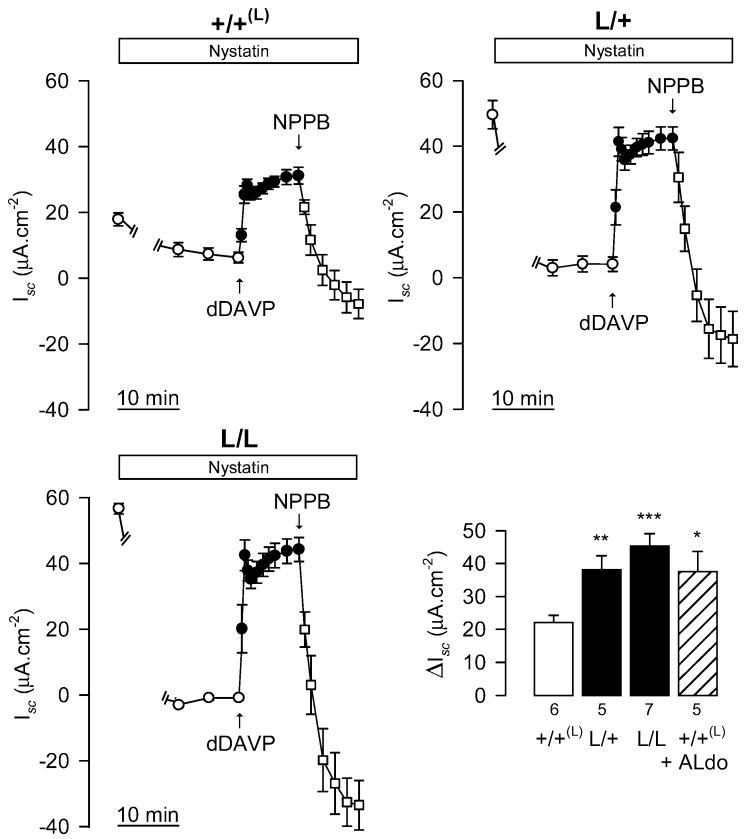

Figure 7. Effects of basolateral membrane permeablilization on dDAVP-stimulated I_{sc}
I_{sc} was measured in sets of confluent cultures of $+/+^{(L)}$, L/+ and L/L CCD cells grown on filters after the basolateral membrane had been permeabilized by adding nystatin (360 μg ml^{-1}) to the basal side of the filter, and a Cl^- gradient (basal medium: 149 mM; apical medium: 14.9 mM) imposed as described in the Methods. Nystatin (○) was added to the basal medium for 20 min. The cells were then sequentially incubated with basal dDAVP (10^{-8} M, ●) for 10 min, and then with apical NPPB (10^{-4} M, □) for a further 10 min. Similar I_{sc} recordings were performed on $+/+^{(L)}$ CCD preincubated with 10^{-6} M aldosterone for 4 h ($+/+^{(L)}$ +Aldo) before permeabilization of the basolateral membrane (hatched bar). Bars represent the relative ΔI_{sc} increase caused by dDAVP. Values are means ± S.E.M. from five to seven separate experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* untreated $+/+^{(L)}$ values.

intercalated cells, which lack ENaC, is not altered by the Scnn1b L and m allele mutations, in contrast to what has been observed in principal cells with vasopressin.

Discussion

The present study provides evidence supporting the existence of a CFTR-dependent Cl^- conductance stimulated by dDAVP that is increased in cultured CCD cells dissected from the kidneys of transgenic L/+ or L/L mice carrying an ENaC mutation causing Liddle's syndrome (Pradervand *et al.* 1999*b*).

Lack of vasopressin-dependent stimulation of Na^+ currents in cultured CCD from Liddle mice

Recent studies have provided strong evidence that ENaC activity in the CCD from L/L mice is still regulated by aldosterone (Auberson *et al.* 2003; Dahlmann *et al.* 2003; Pradervand *et al.* 2003). Snyder (2000) reported that cAMP produced a slight increase or even a decrease in Ams I_{sc} in Fisher rat thyroid cells transfected with the COOH-terminal truncated α- and γ- or β-ENaC mutants. Dahlmann *et al.* (2003) using the same model of Liddle syndrome mice as in the present study have reported that short-term application of cAMP induces a similar increase in amiloride-sensitive I_{Na} currents in microdissected CCD tubules from wild-type and L/L mice. Auberson *et al.* (2003) have shown that vasopressin rapidly increases I_{sc} in cultured renal mpkCCD$_{cl4}$ cells expressing ENaC variants with missense mutation in the PY motif of the β-ENaC subunit or with COOH-terminal truncated β- or γ-ENaC mutants. In this study, the magnitude as well as the rate of the amiloride-sensitive current increase induced by vasopressin was no different in cells expressing wild-type ENaC and Liddle syndrome mutants. Here we found that dDAVP did not result in a significant increase in Ams I_{sc} in L/+ and L/L CCD cells. However, it should be pointed out that the rise in I_{sc} caused by dDAVP is relatively slight ($\sim$10–15 μA cm^{-2}), and one cannot exclude the possibility that this effect may be masked by the fluctuations in the baseline I_{sc} observed in L/L cells before the dDAVP was added (see Fig. 4). It should be pointed out that results obtained in cultured cell systems, such as established cell lines overexpressing ENaC variants, or primary cultures derived from transgenic mice, do not necessarily reflect *in vivo* situations. Dahlmann *et al.* (2003) have shown that I_{Na} in dissected CCD tubules from L/L mice remained low, and were not different from those found in wild-type littermates fed a normal Na^+ diet, but were much greater when the L/L mice were fed a low-Na^+ or a high-K^+ diet or treated with aldosterone. Thus, in L/L mice, the delayed response of aldosterone on Na^+

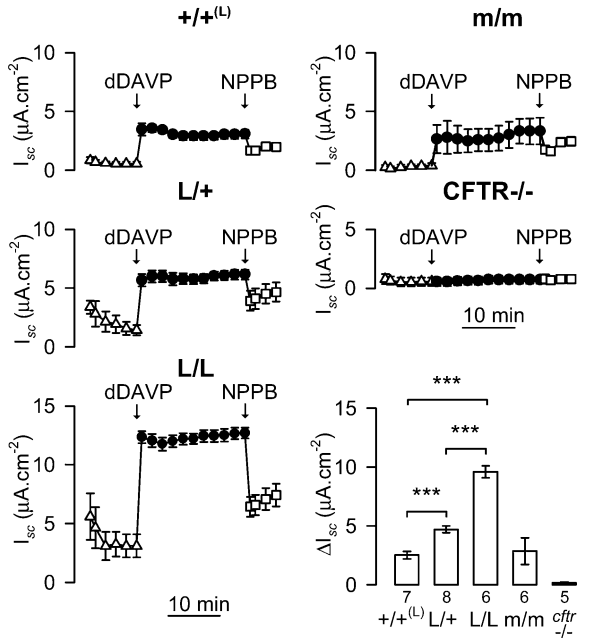

Figure 8. Effects of apical Na^+ substitution on I_{sc} in dDAVP-stimulated cultured CCD cells
I_{sc} was measured in confluent cultures of +/+$^{(L)}$, L/+, L/L, m/m and CFTR−/− (*cftr*−/−) CCD cells grown on filters, and incubated with apical Na^+-free solution (Na^+ was replaced by *N*-methyl-D-glucamine) and basal HFM. After equilibration of the traces, amiloride (Am, 10^{-5} M) was added to the apical side of the cells (Δ). Thereafter, the cells were sequentially incubated with basal dDAVP (10^{-8} M, ●) and apical NPPB (10^{-4} M, □). Bars represent the relative ΔI_{sc} increase caused by dDAVP. Values are means ± S.E.M. from five to eight separate experiments. ***$P < 0.001$ between groups.

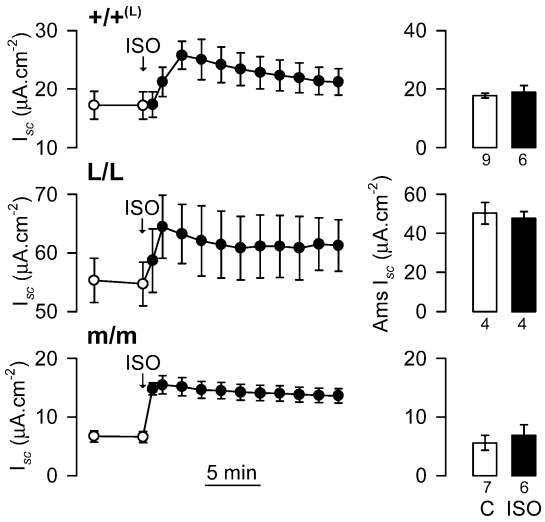

Figure 9. Effects of isoproterenol on I_{sc} in wild-type, L/L and m/m CCD cells
I_{sc} was measured in confluent cultures of +/+$^{(L)}$, L/L and m/m CCD cells grown on filters before (○) and after adding basal isoproterenol (ISO; 10^{-5} M, ●). Amiloride (Am, 10^{-5} M) was then added to the apical side of untreated or ISO-treated cells (not shown) in order to measure Ams I_{sc}. Bars represent Ams I_{sc} values from untreated and ISO-treated cells. Values are means ± S.E.M. from four to nine separate experiments.

absorption (8–9 days) is potentiated by the retention of active ENaC at the cell surface (Dahlmann *et al.* 2003). Acute application of cAMP in CCD dissected from L/L mice and wild-type littermates produced similar increases in ENaC activity after 30 min, but to a much smaller extent than that produced by aldosterone (Dahlmann *et al.* 2003). This suggests that during short periods of cAMP stimulation, the newly active ENaC mutants that cause Liddle's syndrome, which have prolonged activity half-life at the cell surface (Auberson *et al.* 2003), do not have time to accumulate at the cell surface, and no difference is observed between CCD tubules from mutant and wild-type mice (Dahlmann *et al.* 2003). This could explain, at least in part, why cultured CCD cells exhibiting high I_{Na} in non-stimulated conditions, would then become less responsive to the action of aldosterone and in the present case, insensitive to the action of dDAVP.

Higher vasopressin-stimulated Cl^- conductance in cultured CCD from Liddle mice

We have previously shown that vasopressin, unlike aldosterone, stimulates Cl^- reabsorption in mpkCCD cells (Duong Van Huyen *et al.* 1998), and we further demonstrated that CFTR located at the apical membranes of cultured CCD cells was involved in the process of Cl^- secretion (Bens *et al.* 2001). Here we show that the dDAVP-stimulated Cl^- secretion is much greater in Scnn1b mutated L/+ and L/L CCD cells than in $+/+^{(L)}$ CCD cells. In addition, the I_{sc} measurements performed with basolateral membrane permeabilization revealed that the dDAVP-stimulated apical CFTR Cl^- conductance was greater in L/+ and L/L CCD cells than in their wild-type counterparts (Fig. 7). Moreover, the stimulation of ENaC-dependent Na^+ absorption by aldosterone in wild-type CCD cells was also associated with an increase in the dDAVP-stimulated apical CFTR Cl^- conductance (Fig. 7). These findings suggest that any increase in Na^+ absorption mediated by ENaC is accompanied by an increase in apical Cl^- conductance, which culminates in the renal CCD from Liddle mice. A similar observation has already been made: Jiang *et al.* (2000) showed that the open probability and the number of CFTR Cl^- channels increased when CFTR was coexpressed with mouse ENaC in *Xenopus* oocytes, suggesting that CFTR was upregulated by ENaC. The mechanism by which the *Scnn1* R_{566}*stop* mutation leads to an increase in the vasopressin-activated CFTR Cl^- conductance has yet to be determined. We can speculate that the high density of ENaC mutant channels at the cell surface somehow alters the retrieval of CFTR Cl^- channels from the cell surface. This in turn suggests that regulatory proteins other than Nedd-4 may be involved in regulating the activity of CFTR and/or ENaC channels. This regulatory protein could be Syntaxin 1A, a t-SNARE protein receptor involved in membrane fusion, which modulates the activity of ion channels, including CFTR and ENaC (Sanexa *et al.* 2000; Ganeshan *et al.* 2002). Further studies are needed to investigate the control of the ENaC-dependent Na^+ flux and the CFTR-dependent Cl^- flux following vasopressin stimulation in the renal CCD.

Heterologous expression studies have reported a reciprocal effect of CFTR on ENaC. CFTR partially inhibits ENaC in *Xenopus* oocytes or cultured cell systems (Stutts *et al.* 1995; Ji *et al.* 2000; Jiang *et al.* 2000). The inhibitory action of CFTR on ENaC activity is probably indirect, and occurs via an increase in the intracellular Cl^- concentration (Briel *et al.* 1998; König *et al.* 2001) or an effect on membrane potential (Nagel *et al.* 2001). However, in our study, blocking the apical Cl^- conductance by NPPB had no permissive effect on the vasopressin-mediated stimulation of amiloride-sensitive Na^+ currents in L/L CCD cells. This is in agreement with a previous report from our laboratory showing that the Ams I_{sc} were similar in primary cultures of CCD with or without CFTR (Bens *et al.* 2001). Xie & Schafer (2004) have shown that the amiloride-sensitive I_{sc} driven by an apical-to-basal Na^+ gradient is progressively inhibited by the increasing cytosolic concentration of Cl^- in renal MDCK cells stably transfected with flagged rat ENaC subunits. Interestingly, these authors also showed that cAMP produced a significant decrease in the intracellular Cl^-, suggesting that the activation of Cl^- secretion by cAMP would lead to the stimulation rather than the inhibition of ENaC. dDAVP did not induce any significant increase in the amiloride-sensitive I_{sc} in either L/+ or L/L CCD cells. Conversely, Hopf *et al.* (1999) showed that activating CFTR by forskolin inhibited the enhanced Na^+ currents formed by various ENaC Liddle mutations expressed in oocytes. These general data still remain difficult to reconcile with our present findings. As an attempt to provide an explanation, we can note that the results from real-time PCR indicate that there was a large excess of the α-ENaC mRNA subunit relative to CFTR in cultured renal CCD cells (Fig. 2). This means that we cannot exclude the possibility that the differences observed depend to some extent at least, on the levels of endogenous expression of both ENaC and CFTR mRNA in intact collecting duct cells as compared to those in the injected oocytes. Finally, as previously observed, the complex interaction between ENaC and CFTR also seems to depend on the cell type: the activation of CFTR inhibits ENaC channels in airways cells (Mall *et al.* 1998) and colonic cells (Kunzelmann & Mall, 2002), whereas it is accompanied by the concomitant activation of ENaC in absorptive sweat gland cells (Reddy *et al.* 1999).

CFTR acts as a mediator of Cl^- absorption in cultured CCD from Liddle mice

Ion fluxes can conveniently be studied in epithelial monolayers using the short-circuit current method that clamps the transepithelial potential to zero. Using this method, we observed chloride secretion attributable to the presence of an apically located CFTR chloride conductance. The orientation of the Cl^- flux under physiological conditions (when the transepithelial potential difference is not clamped) would be at variance with the experimental condition of short-circuit current, in particular in the CCD cells from Liddle mice. For these cells, which have hyperactive ENaC, the transepithelial potential difference would be sufficiently negative to drive Cl^- absorption under open-circuit conditions in the presence of vasopressin, which is known to act synergistically with aldosterone (Verrey, 1994; Duong Van Huyen *et al.* 1998). Thus, this study provides evidence that renal collecting duct cells from a mouse model for Liddle syndrome exhibit hyperactive vasopressin-regulated CFTR Cl^- conductance, designed to match the increased absorption of sodium.

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