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Glucocorticoid regulation of genes in the amiloride-sensitive sodium transport pathway by semicircular canal duct epithelium of neonatal rat

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Pondugula, Satyanarayana R., Nithya N. Raveendran, Zuhal Ergonul, Youping Deng, Jun Chen, Joel D. Sanneman, Lawrence G. Palmer, and Daniel C. Marcus. Glucocorticoid regulation of genes in the amiloride-sensitive sodium transport pathway by semicircular canal duct epithelium of neonatal rat. *Physiol Genomics* 24: 114–123, 2006. First published November 1, 2005; doi:10.1152/physiolgenomics.00006.2005.—The lumen of the inner ear has an unusually low concentration of endolymphatic Na^+, which is important for transduction processes. We have recently shown that glucocorticoid receptors (GR) stimulate absorption of Na^+ by semicircular canal duct (SCCD) epithelia. In the present study, we sought to determine the presence of genes involved in the control of the amiloride-sensitive Na^+ transport pathway in rat SCCD epithelia and whether their level of expression was regulated by glucocorticoids using quantitative real-time RT-PCR. Transcripts were present for α-, β-, and γ-subunits of the epithelial sodium channel (ENaC); the α_1-, α_3-, β_1-, and β_3-isoforms of Na^+-K^+-ATPase; inwardly rectifying potassium channels [IC_{50} of short circuit current (I_{sc}) for Ba^{2+}: 210 μM] Kir2.1, Kir2.2, Kir2.3, Kir2.4, Kir3.1, Kir3.3, Kir4.1, Kir4.2, Kir5.1, and Kir7.1; sulfonyl urea receptor 1 (SUR1); GR; mineralocorticoid receptor (MR); 11β-hydroxysteroid dehydrogenase (11β-HSD) types 1 and 2; serum- and glucocorticoid-regulated kinase 1 (Sgk1); and neural precursor cell-expressed developmentally downregulated 4-2 (Nedd4-2). On the other hand, transcripts for the α_4-subunit of Na^+-K^+-ATPase, Kir1.1, Kir3.2, Kir3.4, Kir6.1, Kir6.2, and SUR2 were found to be absent, and I_{sc} was not inhibited by glibenclamide. Dexamethasone (100 nM for 24 h) not only upregulated the transcript expression of α-ENaC (~4-fold), β_2-subunit (~2-fold) and β_3-subunit (~8-fold) of Na^+-K^+-ATPase, Kir2.1 (~5-fold), Kir2.2 (~9-fold), Kir2.4 (~3-fold), Kir3.1 (~ 3- fold), Kir3.3 (~2-fold), Kir4.2 (~3-fold), Kir7.1 (~2-fold), Sgk1 (~4-fold), and Nedd4-2 (~2-fold) but also downregulated GR (~3-fold) and 11β-HSD1 (~2-fold). Expression of GR and 11β-HSD1 was higher than MR and 11β-HSD2 in the absence of dexamethasone. Dexamethasone altered transcript expression levels (α-ENaC and Sgk1) by activation of GR but not MR. Proteins were present for the α-, β-, and γ-subunits of ENaC and Sgk1, and expression of α- and γ-ENaC was upregulated by dexamethasone. These findings are consistent with the genomic stimulation by glucocorticoids of Na^+ absorption by SCCD and provide an understanding of the therapeutic action of glucocorticoids in the treatment of Meniere's disease.

inner ear; vestibular labyrinth; dexamethasone

WE HAVE RECENTLY SHOWN that the semicircular canal duct (SCCD) epithelium contributes to the low level of Na^+ in vestibular endolymph. Na^+ absorption is mediated via amiloride-sensitive epithelial sodium channels (ENaC) in the apical membrane under glucocorticoid control via glucocorticoid receptors (GR) (41). Hypoabsorption of Na^+ from the vestibular lumen has been suggested to be associated with endolymphatic hydrops, a manifestation of the debilitating condition known as Meniere's disease (33, 50).

It has been shown that vectorial transport of Na^+ by SCCD epithelium from the lumen into the perilymph requires not only ENaC at the apical membrane but also the involvement of ouabain-sensitive Na^+-K^+-ATPase and Ba^{2+}-sensitive potassium channels at the basolateral membrane (41). However, isoforms of the cation transporters and regulatory proteins involved in transepithelial Na^+ transport by SCCD epithelia are not known.

ENaC is a heteromultimeric channel composed of α-, β-, and γ-subunits (1, 25). Recently, a δ-subunit of ENaC was also cloned from the human brain (57). Apical Na^+ from the endolymph enters SCCD epithelial cells through ENaC and is extruded from the cytosol into the perilymph across the basolateral membrane by Na^+-K^+-ATPase, which is a heterodimer composed of one α-subunit and one β-subunit. Four α-subunit (α_1, α_2, α_3, and α_4) and four β-subunit (β_1, β_2, β_3, and β_4) isoforms of Na^+-K^+-ATPase have been identified (39, 40, 52). Recently, a γ-subunit was also cloned and found to regulate the function of Na^+-K^+-ATPase (2).

The function of Na^+-K^+-ATPase depends on the presence of a K^+ "leak" in the basolateral membrane. Indeed, the dexamethasone (Dex)-stimulated short circuit current (I_{sc}) across SCCD is partially inhibited by Ba^{2+}, a K^+ channel blocker. Inward rectifier K^+ channels (Kir channels) are highly sensitive to Ba^{2+} and are classified into seven subfamilies (Kir1–Kir7), with some subfamilies having several isoforms (7).

Neural precursor cell-expressed developmentally downregulated 4-2 (Nedd4-2) and serum- and glucocorticoid-regulated kinase 1 (Sgk1) are known to regulate the expression of ENaC in many mammalian epithelial tissues (53). Nedd4-2 decreases Na^+ absorption by reducing the expression of ENaC in the apical membrane (51, 53). In contrast to Nedd4-2, Sgk1 increases Na^+ absorption by increasing the expression of ENaC in the apical membrane via inactivation of Nedd4-2 (51, 53).

It has been shown in mammalian epithelia and expression systems that genomic stimulation of vectorial Na^+ transport by glucocorticoids involves an altered transcript expression of cation transporters such as ENaC subunits (8, 21, 37) and Na^+-K^+-ATPase (3, 8, 18, 34) and Kir isoforms (15).

It has also been shown that glucocorticoids regulate the transcript expression of the regulatory proteins Sgk1 (21, 36)

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and GR (11, 20, 28, 58) and glucocorticoid metabolism-regulatory enzymes [11β-hydroxysteroid dehydrogenase (11β-HSD) isoforms (22)], which in turn control epithelial Na^+ transport. Because glucocorticoids increase GR-dependent vectorial Na^+ transport by SCCD epithelium (41), we sought to determine whether genes encoding for cation transporters, corticosteroid receptors, and key regulatory proteins are expressed and whether their levels are regulated by glucocorticoids in SCCD epithelium.

Our findings of glucocorticoid-regulation of genes involved in Na^+ transport are consistent with glucocorticoid-stimulation of Na^+ transport by SCCD epithelia and provide a basis of molecular action of therapeutic glucocorticoids at the transcriptional level for treatment of Meniere's disease.

MATERIALS AND METHODS

Primary cultures of SCCD epithelium. Epithelial cells from the semicircular canals of neonatal (*days 3–5*) Wistar rats, excluding the common crus, were dispersed and seeded on 6.5-mm-diameter Transwell permeable supports (Costar no. 3470, Corning) and cultured in DMEM-F-12 medium supplemented with 5% FBS as described previously (31). Cultures treated with 100 nM Dex [cyclodextrin-encapsulated Dex (no. D-2915, Sigma) dissolved in water] in the presence and absence of either the GR antagonist mifepristone (M-8046, Sigma, dissolved in DMSO) or mineralocorticoid receptor (MR) antagonist spironolactone (no. S-3378, Sigma, dissolved in DMSO) were exposed for 24 h followed by RNA isolation. Some cultures were treated with 0.1% DMSO (no. D-2650, Sigma) for 24 h followed by RNA isolation as a control for studies of long-term exposure to hydrophobic drugs predissolved in DMSO (41).

RNA isolation. Total RNA was extracted from untreated, Dex-treated, Dex and antagonist-treated, and DMSO-treated SCCD primary culture cells using a RNeasy Micro Kit following the manufacturer's protocol (no. 74004, Qiagen; Valencia, CA). RNA for positive controls was obtained from the rat brain (RNA was extracted using the RNeasy Micro Kit following the manufacturer's instructions) or rat kidney (no. 7926, Ambion; Austin, TX). Total RNA quality was determined with an Agilent BioAnalyzer (model 2100, Agilent; Palo Alto, CA; Fig. 1*A*), and quantity was determined with either the BioAnalyzer or a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies; Fig. 1*B*).

Analysis of gene expression by quantitative real-time RT-PCR. Real-time RT-PCR was performed on total RNA obtained from SCCD primary cultures using 0.2× SYBR green 1 (no. S7567, Molecular Probes), a One Step RT-PCR Kit (no. 210210, Qiagen), and a Smart Cycler thermocycler (Cepheid; Sunnyvale, CA). Transcripts of 18S rRNA and target genes were amplified using gene-specific primers (Table 1). Reverse transcription (RT) was performed for 30 min at 50°C and 15 min at 95°C. Each of the 50 PCR cycles was composed of 95°C for 1 min, 55°C (65°C for α_2-Na^+-K^+-ATPase and Kir2.1; 60°C for 18S rRNA, Kir2.2, Kir2.4, Kir4.1, Kir4.2, Kir5.1, and Kir7.1; and 63°C for Kir3.3) for 1 min, and 72°C for 1 min. PCR was followed by a melt at 60–95°C. Sample fluorescence was read at 2–3°C below the melting temperature peak of each specific cDNA to exclude contributions from nonspecific sources. To exclude the possibility of genomic DNA amplification during the PCR reaction, RT negative controls (−RT) were performed and accepted when the threshold value (C_t) was at least nine cycles greater than the +RT run. PCR products were run on 2% agarose gels and detected by ethidium bromide. PCR products were then purified by using either a gel extraction kit (no. 28704, Qiagen) or PCR purification kit (no. 28104, Qiagen), and purified PCR products were sequenced to verify the identity of the RT-PCR products. The specific gene expression was normalized to the level of 18S rRNA in each sample as described previously (59, 62), taking into account the fidelity of each PCR. Template molecules for 18S rRNA and genes of interest were determined as described previously (59, 62).

For qualitative gene expression studies (Kir1.1, Kir3.2, Kir3.4, Kir6.1, Kir6.2, SUR1, and SUR2), RT was performed on 10 ng of total RNA followed by 40 PCR cycles. Each PCR cycle was composed of 95°C for 1 min, 55°C (60°C for Kir1.1 and SUR1) for 1 min, and 72°C for 1 min. PCR products were run on 2% agarose gels and detected by ethidium bromide. Purified PCR products were sequenced to verify the identity of the RT- PCR products.

A series of experiments (see Fig. 7) to test for specificity of the steroid receptor involved in stimulated transcript expression levels were carried out using an iCycler iQ Real-Time PCR Detection System (Bio-Rad). RT was performed as described above followed by 40 PCR cycles using an annealing temperature of 60°C for 1 min, and the rest of the procedure was the same as above. The fidelity of each PCR was calculated using LinReg PCR software (44). Quantification of template molecules for 18S rRNA and genes of interest were determined as described previously (59, 62).

Western blot analysis of ENaC subunits and Sgk1. SCCD primary cultures were grown on 12-mm-diameter Snapwell permeable supports (Costar no. 3801, Corning). Each of the Dex-treated and untreated confluent SCCD monolayers were washed in PBS (150 mM NaCl, 8 mM Na_2HPO_4 $2H_2O$, and 2 mM KH_2PO_4; pH 7.4) at room temperature and then lysed with trituration in about 75 μl of cold (4°C) radioimmunoprecipitation (RIPA) buffer (10 mM Tris base, 1% sodium deoxycholate, 1% Nonidet P-40, and 150 mM NaCl; pH 7.9) containing protease inhibitor cocktail (no. P-2714, Sigma). Lysates harvested from three permeable supports of the same condition were pooled. Supernatants from whole cell lysates were collected after centrifugation at 15,000 rpm for 5 min at 4°C, and the total protein concentration was determined using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies).

To evaluate the protein expression of ENaC subunits in SCCD, about 40 μg of total protein were solubilized at 70°C for 10 min in Laemmli sample buffer and resolved on 4–12% bis-Tris gels (Invitro-

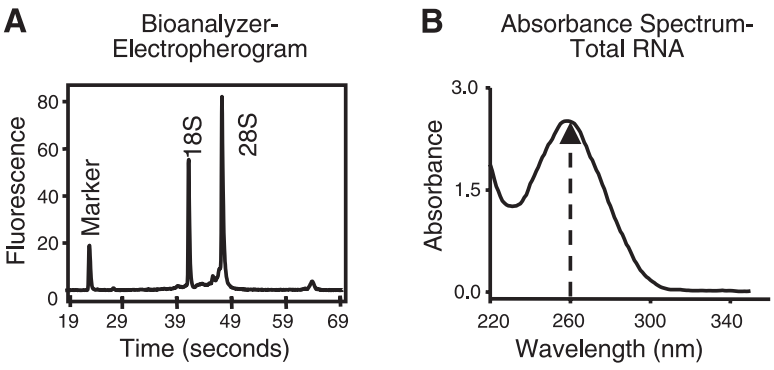

Fig. 1. Representative displays of quality and quantity of total RNA obtained from primary cultures of semicircular canal duct (SCCD) epithelium. *A*: electropherogram from the Agilent BioAnalyzer of SCCD total RNA. The quality and quantity of total RNA and 18S rRNA was determined as described previously (59, 62). *B*: absorption spectrum from the NanoDrop spectrophotometer. The smooth absorbance spectra with an absorbance maxima at 260 nm (dashed arrow) demonstrate the high quality of total RNA. The amount of total RNA was obtained from the amount of light absorbed by total RNA.

Table 1. *Primers for quantitative and qualitative RT-PCR analysis*

Protein (Gene)	Gene Bank Accession No.	Forward Primer	Reverse Primer	Amplicon Size, bp
18S	BK000964	5′-GAGGTTCGAAGACGATCAGA-3′	5′-TCGCTCCACCAACTAAGAAC-3′	315
α-ENaC	NM_031548	5′-CTAGACCTTCACGCCTCCAAC-3′	5′-AGGTACTCCTCGAACAGCAAG-3′	365
β-ENaC	NM_012648	5′-TGCCAATGGGACCGTGTGTA-3′	5′-GCCCCAGTTGAAGATGTAGC-3′	244
γ-ENaC	NM_017046	5′-ACAAAGACCTGAACCAAAG-3′	5′-GCATAGCAGAGGTAAAAGT-3′	305
α_1-Na^+-K^+-ATPase	NM_012504	5′-TCCTCCCTCTTTCCTCCG-3′	5′-GCCTCGGCTCAAATCTGTTC-3′	325
α_2-Na^+-K^+-ATPase	NM_012505	5′-GGGCCTGACTAATTTGAGATCACTG-3′	5′-GTCTCACAGAAGGTCACCAGTAAGG-3′	254
α_3-Na^+-K^+-ATPase	NM_012506	5′-CCACACCTCGGTTACCTCTCAC-3′	5′-CAGATTTAGAACCGGAGATGGC-3′	206
α_4-Na^+-K^+-ATPase	NM_022848	5′-ACAACTTTGCCTCCATTGTGAC-3′	5′-TTCATGCCCTGCTTGAAGAGT-3′	592
β_1-Na^+-K^+-ATPase	BC_027319	5′-CATCTGGAACTCGGAGAAGAAGG-3′	5′-TTGGGGTCATTAGGACGGAAG-3′	243
β_2-Na^+-K^+-ATPase	J04629	5′-TTGGGAAGAAAGATGAAGAT-3′	5′-AGGAGGGTATGGGTGAGAGG-3′	304
β_3-Na^+-K^+-ATPase	NM_012913	5′-GCTCCCAATCCCAAACAT-3′	5′-CGACAGGCACTCACTCACAC-3′	294
Kir1.1 (KCNJ1)	NM_017023	5′-ACCAAGGAAGGGAAATACCG-3′	5′-GTTCAGGGGTGACTGTGCTA-3′	310
Kir2.1 (KCNJ2)	NM_017296	5′-TGGGAGAGAAAGGACAGAGG-3′	5′-AAAACAGCAATCGGGCACT-3′	295
Kir2.2 (KCNJ12)	NM_053981	5′-GGCATCATCTTCTGGGTCAT-3′	5′-AGTCTGTGCTCGCTTCTTGG-3′	285
Kir2.3 (KCNJ4)	X87635	5′-CAGGCCCACGTGCCCAGGCGGA-3′	5′-TACATGCATGATACACGGTTTG-3′	330
Kir2.4 (KCNJ14)	NM_170718	5′-GTGCCTGCTCTTCTCCTGTT-3′	5′-GTGTCTCGTTGCGTTTCTTG-3′	326
Kir3.1 (KCNJ3)	NM_031610	5′-GCACCAGCCATAACCAAC-3′	5′-TTGCCAGGAACCGAACTT-3′	221
Kir3.2 (KCNJ6)	X83583	5′-CGTGGCTTTTCTTTGGGATG-3′	5′-CCTCTTCTTGGGTTGGGATA-3′	290
Kir3.3 (KCNJ9)	NM_053834	5′-CCTTGACGCCCATCTCTACT-3′	5′-CCCCACCCTCTATTTTCCTC-3′	341
Kir3.4 (KCNJ5)	NM_017297	5′-GGCTTTGTGTCTGCTTTCCT-3′	5′-ATTCCCCTTCTTTGGTCTGC-3′	343
Kir4.1 (KCNJ10)	NM_031602	5′-GCCCCTTTCTCATTCTACCC-3′	5′-GACGCCACTTTCACAACCTG-3′	277
Kir4.2 (KCNJ15)	NM_133321	5′-GGTTTCTTTTTGGGGTGGTC-3′	5′-GCTCGCTTTTTGGGTCTTG-3′	292
Kir5.1 (KCNJ16)	AF249676	5′-TCCTCTGTATGCCCTTGACC-3′	5′-ATCTCCTCCTGGTGTTGGTG-3′	338
Kir6.1 (KCNJ8)	NM_017099	5′-GAGTGAACTGTCGCACCAGA-3′	5′-CGATCACCAGAACTCAGCAA-3′	248
Kir6.2 (KCNJ11)	X97041	5′-CTGCCTTCCTTTTCTCCATC-3′	5′-TTACCACCCACACCGTTCTC-3′	385
Kir7.1 (KCNJ13)	AJ292748	5′-CAGCCCCCACATAGATACAC-3′	5′-TAACCACTTCCTTCCCTCCT-3′	292
SUR1 (ABCC8)	AJ511273	5′-TGAAGCAACTGCCTCCATC-3′	5′-GAAGCTTTTCCGGCTTGTC-3′	182
SUR2 (ABCC9)	D83598	5′-TGAAAATAACCTGAAGCCCG-3′	5′-CCTGGCAGAGATTTGACCAT-3′	345
Sgk1	NM_019232	5′-CTGCAATGTGCCTTTTCTGA-3′	5′-ATGCTTCCCTCAAGCATCTG-3′	352
Nedd4-2	XM_214557	5′-GCTGGGAAGAAAGAAAAGATG-3′	5′-TTGGGGGAGTTGTAAGGTGA-3′	298
GR	NM_012576	5′-AAAATGGGTCGGTGCTTCTA-3′	5′-TTACGGGCTTGGTTGCTATC-3′	319
MR	NM_013131	5′-AGAAGATGCATCAGTCTGCC-3′	5′-GTGATGATCTCCACCAGCAT-3′	382
11β-HSD1	NM_017080	5′-AAATACCTCCTCCCCGTCCT-3′	5′-TCCTGCCTCAACAACAAATC-3′	303
11β-HSD2	NM_017081	5′-CAAACCCTTCCCCCACAG-3′	5′-GCCACATCTCACGCTAAACTC-3′	301

18S, 18S small subunit rRNA; ENaC, epithelial sodium channel; Kir, inwardly rectifying potassium channel; SUR, sulfonyl urea receptor; Sgk1, serum- and glucocorticoid-regulated kinase 1; Nedd4-2, neural precursor cell-expressed developmentally downregulated 4-2; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; 11β-HSD, 11β-hydroxysteroid dehydrogenase.

gen) by SDS-PAGE. For immunoblot analysis, proteins were transferred electrophoretically from unstained gels to polyvinylidene difluoride membranes. After being blocked with BSA, membranes were incubated overnight at 4°C with primary antibodies against α-, β-, and γ-subunits [rabbit polyclonal antibodies against the α-, β-, and γ-subunits of rat ENaC were generated by Lawrence G. Palmer as described previously (29)] at 1:500 or 1:1,000 dilutions. Anti-rabbit IgG conjugated with alkaline phosphatase was used as a secondary antibody. The sites of antibody-antigen reaction were visualized with a chemiluminescence substrate (Western Breeze, Invitrogen) before exposure to X-ray film (Kodak, Biomax ML).

To evaluate the protein expression for Sgk1 in SCCD, about 30 μg of total protein were diluted in Laemmli sample buffer (no. 161-0737, Bio-Rad) containing 5% 2-mercaptoethanol (no. M-7154, Sigma), boiled for 10 min at 70°C, and separated using a 4–15% Tris·HCl precast polyacrylamide gel (no. 161-1104, Bio-Rad, 150 V for 50 min in 25 mM Tris base, 192 mM glycine, and 0.1% SDS). Proteins were transferred to a nitrocellulose membrane (no. 162-0114, Bio-Rad) using the Trans-Blot SD Semi-Dry Electrophoretic System (Bio-Rad, 15 V for 45 min in 25 mM Tris base, 192 mM glycine, and 20% methanol; pH 8.3). Membranes were then blocked with 5% nonfat dry milk [no. 170-6404, Bio-Rad, in 20 mM Tris base, 137 mM NaCl, and 0.1% Tween 20 (TBS); pH 7.6] for 1 h and then probed for 1 h with the primary antibody against Sgk1 (rabbit polyclonal anti-Sgk1, no. S-5188, Sigma) at a dilution of 1:2,000 in blocking buffer. Horseradish peroxidase-conjugated secondary donkey anti-rabbit IgG (no. NA934V, Amersham Biosciences) was diluted to 1:20,000 in blocking buffer and then used to incubate the membrane for 1 h. Specific bands were visualized with a chemiluminescent substrate (nos. 34080 and 34095; Pierce, Rockford, IL). The same membranes that were probed for anti-Sgk1 were stripped using Restore Western Blot Stripping Buffer (no. 21059, Pierce) and then reprobed with anti-actin antibody to confirm equal loading (data not shown). Equal loading and protein quality were assessed by gel staining with Bio-Safe Coomassie Stain (no. 161-0786, Bio-Rad). Equal loading and protein transfer were confirmed by Ponceau S (no. P-7170, Sigma) staining of the membranes. All steps were carried out at room temperature.

Electrophysiological measurements. SCCD epithelial monolayers were bathed in symmetric HEPES-buffered solution equilibrated with air for electrophysiological experiments with glibenclamide [Glib; G-0639, Sigma, dissolved in DMSO) and Ba^{2+} ($BaCl_2$ dihydrate, no. 11760, Fluka Chemica, dissolved in water). The composition of the HEPES-buffered solution was (in mM) 150 NaCl, 3.6 KCl, 1 $MgCl_2$, 0.7 $CaCl_2$, 5 glucose, and 10 HEPES, pH 7.4.

Transepithelial voltage (V_T) and resistance (R_T) were measured from confluent monolayers of SCCD in an Ussing chamber (AH 66-0001, Harvard Apparatus, Holliston, MA) modified to accept the 6.5 mm Transwells, maintained at 37°C, and connected to a voltage-current clamp amplifier (model VCC600, Physiologic Instruments; San Diego, CA) via Ag/AgCl electrodes and HEPES-buffered bath solution bridges with 3% agar. V_T and R_T were measured under current clamp (change in current = 1 μA), and the equivalent I_{sc} was calculated from $I_{sc} = V_T/R_T$. Apical and basolateral side baths were stirred by air. Ba^{2+} was added to the basolateral bath cumulatively.

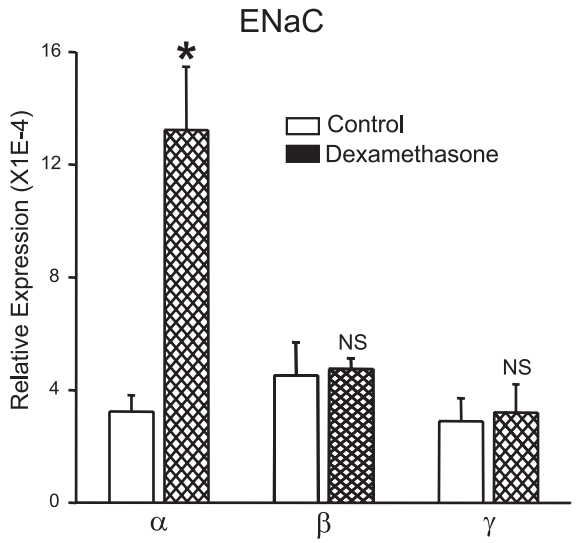

Fig. 2. Real-time RT-PCR evaluation of the transcript expression of α-, β-, and γ-subunits of the epithelial sodium channel (ENaC) in SCCD epithelia before and after dexamethasone (Dex; 100 nM for 24 h) application. Values are expressed relative to 18S rRNA transcript expression (means ± SE); n = 6. NS, not significant. *$P < 0.05$, without vs. with Dex.

Glib (20 μM) was first added to the basolateral bath and then to the apical bath to separately evaluate the basolateral and apical effects.

Statistical analyses. Real-time RT-PCR data were normalized by taking the ratio of the amount of transcript expression of the target gene to the amount of transcript expression of 18S rRNA (59, 62). Relative expression was then logarithmically transformed to determine statistical significance of unpaired samples using Student's *t*-test. Relative expression is shown as mean values ± SE from *n* observations. Differences were considered significant for $P < 0.05$. The Hill equation was fitted to the Ba^{2+} concentration-response curve by using individual data points to retain appropriate weighting and presented here plotted with the mean and SE for clarity.

RESULTS

Glucocorticoid upregulation of the transcripts for cation transporters. Our recent functional studies of I_{sc} measurements demonstrated that SCCD epithelia absorb Na^+ via ENaC at the apical membrane upon stimulation by glucocorticoids (41). Qualitative RT-PCR results demonstrated expression of the transcripts for all three ENaC subunits in SCCD epithelia both under glucocorticoid-treated and untreated conditions (41). We investigated here whether Dex regulates the transcript expression of ENaC subunits in SCCD epithelia. The transcript expression for α-subunit of ENaC was upregulated by approximately fourfold, whereas the β- and γ-subunits were not significantly altered after Dex application (Fig. 2). The relative abundance of the transcripts for ENaC subunits in the absence of Dex was found to be not significantly different among the three subunits (Fig. 2).

Na^+-K^+-ATPase and Ba^{2+}-sensitive potassium channels are involved in glucocorticoid-stimulated Na^+ transport at the basolateral membrane in SCCD epithelia to extrude cytoplasmic Na^+ and recycle K^+, respectively, across the basolateral membrane and into the perilymph (41). We investigated whether transcripts for isoforms of Na^+-K^+-ATPase and Ba^{2+}-sensitive Kir are expressed and whether their levels are altered by Dex in SCCD epithelia. Transcripts for the α_1-, α_3-, β_1-, and β_3-subunits of Na^+-K^+-ATPase (Fig. 3) were found to be expressed. However, the transcripts for the α_4-subunit (Fig. 3, *inset*) were found to be absent in SCCD epithelia, and trace amounts of the α_2- and β_2-subunits were detected. It was also found that the transcripts for the β_2- and β_3-subunits of Na^+-K^+-ATPase were upregulated significantly by approximately two- and eightfold, respectively, after Dex incubation (Fig. 3). The relative abundance of the transcripts for Na^+-K^+-ATPase isoforms in the absence and presence of Dex was found to be in the order of $\alpha_1 = \beta_1 > \alpha_3 = \beta_3 > \beta_2 > \alpha_2$ and $\beta_3 > \beta_1 = \alpha_1 > \alpha_3 > \beta_2 > \alpha_2$, respectively (Fig. 3).

Even though Ba^{2+} is a broad-spectrum potassium channel blocker, it selectively blocks Kir channels with an apparent K_i of ~100 μM (26). Ba^{2+} blocked Dex (100 nM for 24 h)-stimulated I_{sc} (under conditions where Cl^- secretion was small and most of the I_{sc} was carried by Na^+) in SCCD epithelia in a concentration-dependent manner with an IC_{50} of ~210 μM (Fig. 4*A*), indicating that Kir channels are involved in Dex-stimulated Na^+ transport. Transcripts for Kir2.1, Kir2.2, Kir2.3, Kir2.4, Kir3.1, Kir3.3, Kir4.1, Kir4.2, Kir5.1, and Kir7.1 were found to be expressed (Fig. 4*E*); however, Kir1.1 (Fig. 4*B*), Kir3.2 (Fig. 4*C*), and Kir3.4 (Fig. 4*D*) were found to be absent. It was also found that the transcripts for Kir2.1, Kir2.2, Kir2.4, Kir3.1, Kir3.3, Kir4.2, and Kir7.1 were upregulated significantly by approximately five-, nine-, three-, three-, two-, three-, and twofold, respectively, after Dex treatment

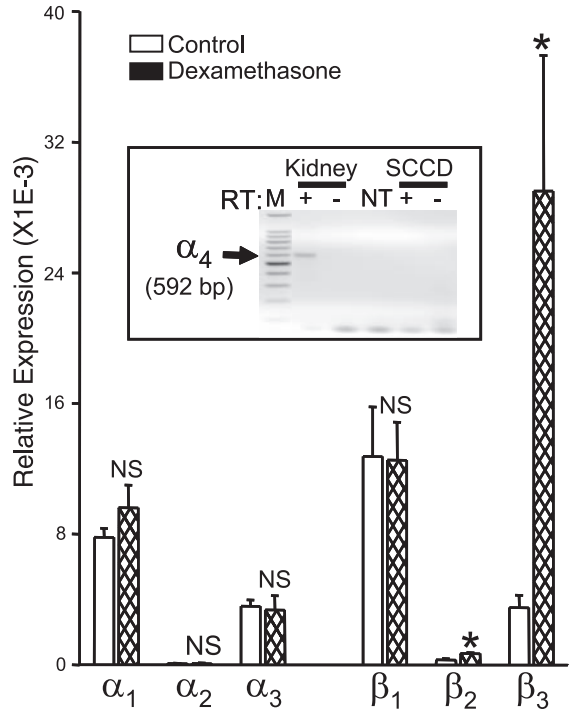

Fig. 3. Real-time RT-PCR evaluation of the transcript expression of α_1-, α_2-, α_3-, β_1-, β_2-, and β_3-isoforms of Na^+-K^+-ATPase in SCCD epithelia before and after Dex (100 nM for 24 h) exposure. Values are expressed relative to 18S rRNA transcript expression (means ± SE); $n = 3$–6. *$P < 0.05$, without vs. with Dex. *Inset*: absence of the transcript for α_4-Na^+-K^+-ATPase in SCCD epithelia. A single band was observed at the expected size [+reverse transcription (RT)] in the rat kidney but not in SCCD primary cultures (+RT). No signal was observed in negative-RT controls (−RT) and no-template control (NT); $n = 3$. Identity of the band was verified by sequence analysis. M, 100-bp ladder.

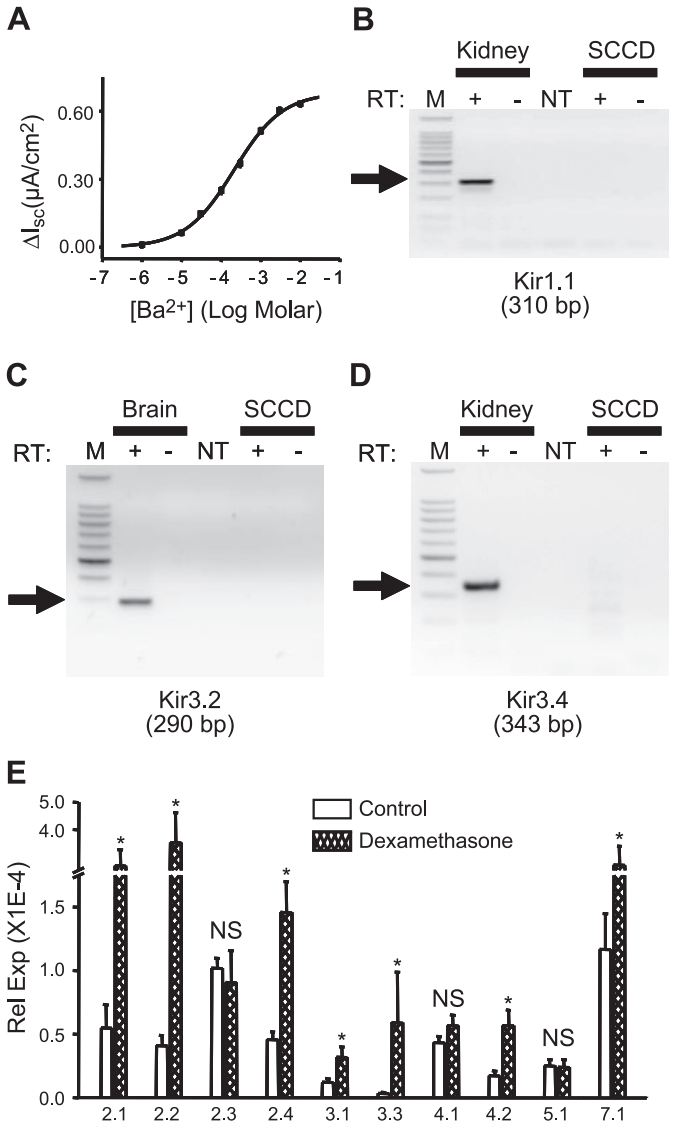

Fig. 4. *A*: concentration-response curve for the decrease of Dex (100 nM for 24 h)-increased short circuit current (I_{sc}) by Ba^{2+}. The curve is the best fit to the Hill equation. The initial value of I_{sc} after Dex stimulation is 8.05 ± 0.48 μA/cm^2. The curve was a Hill function with best-fit parameters of V_{max} = 0.68, Hill coefficient = 0.7, IC_{50} = 210 μM. Data are means ± SE; n = 5. *B–D*: absence of transcripts for Kir1.1 (*B*), Kir3.2 (*C*), and Kir3.4 (*D*) in SCCD epithelia. Arrows indicate the size of the target genes. Single bands were observed at the expected size (+RT) in the rat brain (for Kir3.2) and rat kidney (for Kir1.1 and Kir3.4) but not in SCCD primary cultures. No signal was observed in −RT and NT. n = 3. Identity of the bands was verified by sequence analysis. *E*: real-time RT-PCR evaluation of the transcript expression of Kir2.1 (2.1; KCNJ2), Kir2.2 (2.2; KCNJ12), Kir2.3 (2.3; KCNJ4), Kir2.4 (2.4; KCNJ14), Kir3.1 (3.1; KCNJ3), Kir3.3 (3.3; KCNJ9), Kir4.1 (4.1; KCNJ10), Kir4.2 (4.2; KCNJ15), Kir5.1 (5.1; KCNJ16), and Kir7.1 (7.1; KCNJ13) isoforms in SCCD epithelia before and after Dex (100 nM for 24 h) application. Values are expressed relative (Rel Exp) to 18S rRNA transcript expression (means ± SE); n = 4. *$P < 0.05$, without vs. with Dex. Note the break in the vertical axis.

(Fig. 4*E*). The relative abundance of the transcripts for Kir isoforms in the absence and presence of Dex is shown in Fig. 4*E*. The Glib-inhibitable and ATP-sensitive Kir6.1 and Kir6.2 channels are heterooctomers consisting of four subunits of either Kir6.1 or Kir6.2 and four subunits of either SUR1 or SUR2. The transcripts for Kir6.1 (Fig. 5*B*), Kir6.2 (Fig. 5*C*), and SUR2 (Fig. 5*D*) were found to be absent in SCCD, and the I_{sc} of Dex-treated SCCD was not affected by Glib (20 μM; Fig. 5*A*). The I_{sc} from these epithelia, however, was sensitive to 3 mM Ba^{2+} (Fig. 5*A*), as observed earlier (Fig. 4*A*) (41).

Glucocorticoid upregulation of transcript expression for Sgk1 and Nedd4-2. The transcripts for both Sgk1 and Nedd4-2 were upregulated after Dex exposure by approximately four- and twofold, respectively (Fig. 6*A*).

Glucocorticoid downregulation of the transcript expression for GR and 11β-HSD1. GR was found to be the predominant corticosteroid receptor in SCCD and was downregulated by approximately threefold after Dex application (Fig. 6*B*). MR expression was comparatively small and was not affected by Dex (Fig. 6*B*).

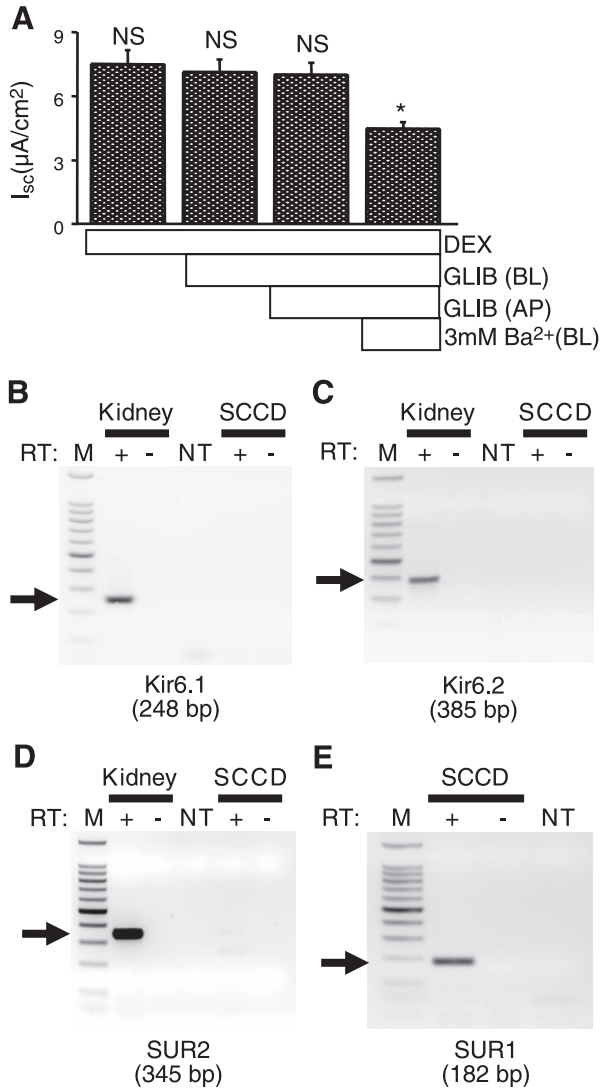

Fig. 5. *A*: effect of apical (AP) and basolateral (BL) glibenclamide (Glib; 20 μM) on Dex (100 nM for 24 h)-stimulated I_{sc}. Data are means ± SE; n = 4. *$P < 0.05$, I_{sc} compared with either Dex alone or Dex and Glib. *B–D*: absence of transcripts for Kir6.1 (*B*), Kir6.2 (*C*), and sulfonyl urea receptor 2 (SUR2; *D*) in SCCD monolayers. Arrows indicate the size of target genes. Single bands were observed at the expected size (+RT) in the rat kidney but not in SCCD primary cultures. *E*: presence of the transcript for SUR1 in SCCD epithelia. A single band, indicated by the arrow, was observed at the expected size (+RT) in SCCD primary cultures. No signal was observed in −RT and NT. n = 3. Identity of the bands was verified by sequence analysis.

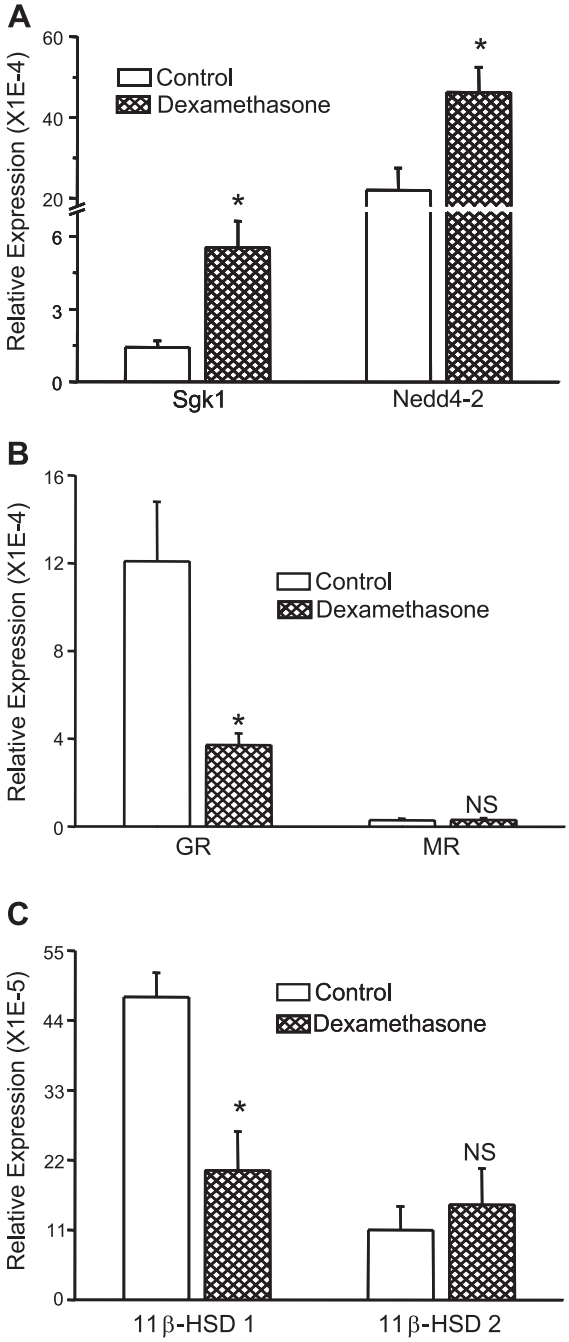

Fig. 6. Real-time RT-PCR evaluation of transcript expression of serum- and glucocorticoid-regulated kinase 1 (Sgk1) and neural precursor cell-expressed developmentally downregulated 4-2 (Nedd4-2) (*A*), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (*B*), and 11β-hydroxysteroid dehydrogenase (11β-HSD) types 1 and 2 (*C*) in SCCD epithelia before and after Dex (100 nM for 24 h) application. Values are expressed relative to 18S rRNA transcript expression (means ± SE); $n = 3–6$. *$P < 0.05$, without vs. with Dex. Note the break in the vertical axis in *A*.

Intracellular 11β-HSD isoforms are glucocorticoid-metabolism enzymes that regulate the intracellular concentration of active glucocorticoids in corticosteroid-responsive target tissues by catalyzing the interconversion of biologically active and inactive intracellular glucocorticoids. 11β-HSD isoforms could regulate Na^+ transport indirectly by controlling the concentration of active intracellular glucocorticoids. It has been found that 11β-HSD1 is expressed in a wide range of tissues (5), whereas 11β-HSD2 is expressed mainly in mineralocorticoid target tissues (5, 13). Because Na^+ transport in SCCD epithelia is under glucocorticoid control, we investigated whether the transcript for 11β-HSD1 is expressed and regulated by Dex in SCCD epithelia. In fact, the transcripts for both 11β-HSD1 and 11β-HSD2 were found to be expressed (Fig. 6*C*).

11β-HSD1 was found to be the predominant enzyme in SCCD and was downregulated by approximately twofold after Dex application (Fig. 6*C*). 11β-HSD2 expression was comparatively small and was not affected by Dex (Fig. 6*C*).

Protein expression of Na^+ transport pathway genes. Western blot analysis demonstrated that protein was present for the α- (85 kDa), β- (85 kDa), and γ- (80 kDa) subunits of ENaC in SCCD (Fig. 7*A*). An additional band (indicated by the gray arrow) that ran a little slower than the one in the absence of Dex was observed with Dex treatment. This is consistent with a different glycosylation pattern of α-ENaC in Dex-treated SCCD. The abundance of total protein expression, including

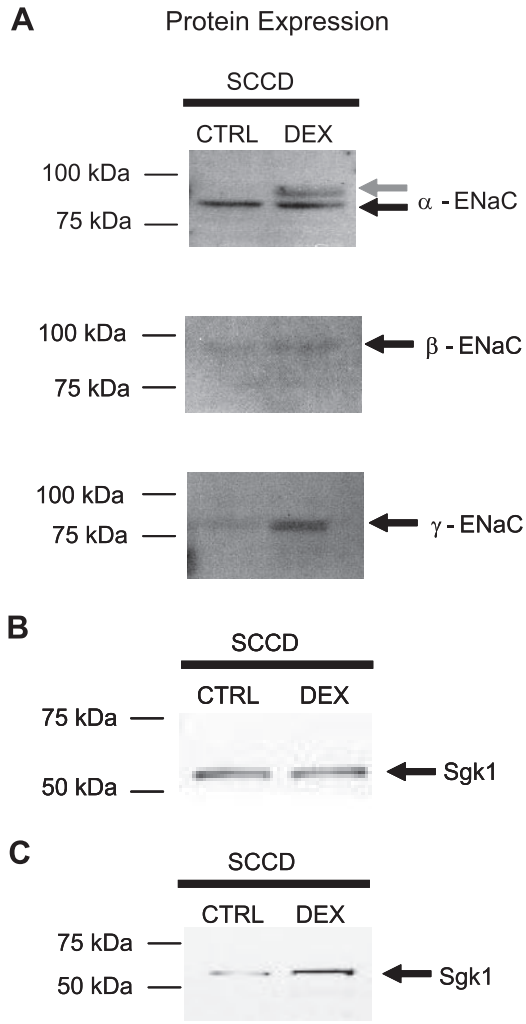

Fig. 7. Protein expression of ENaC subunits and Sgk1 in SCCD epithelia. Representative Western blots for subunits of ENaC (*A*) and Sgk1 (*B* and *C*) are shown. The gray arrow, in *A*, *top*, indicate the differently glycosylated form of the α-subunit of ENaC in Dex-treated SCCD primary cultures. CTRL, control (untreated) condition. $n = 2–3$ each.

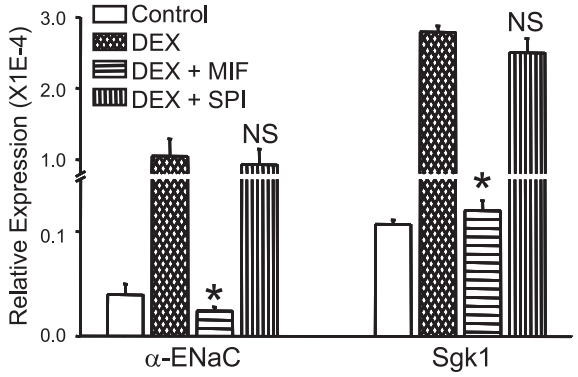

Fig. 8. Glucocorticoids act at GR to regulate the transcript expression of α-ENaC and Sgk1. Real-time RT-PCR was performed on RNA obtained from primary cultures of SCCD epithelia under control, Dex (100 nM), Dex + GR antagonist mifepristone (Mif; 100 nM), and Dex + MR antagonist spironolactone (Spi; 10 μM) conditions. Values are expressed relative to 18S rRNA transcript expression (means ± SE); $n = 4$. *$P < 0.05$, Dex vs. Dex + antagonist. Cultures were exposed to conditions for 24 h. Note the break in the vertical axis.

the additional band, for α-ENaC was greater with Dex treatment. Similarly, the abundance of total protein for the γ-subunit of ENaC was higher with Dex treatment, suggesting that Dex upregulates both the α- and γ-subunits of ENaC in SCCD. There was no apparent change in β-subunit ENaC protein expression.

Sgk1 protein was expressed in SCCD (Fig. 7, *B* and *C*). Three sample sets each of Western blots (Fig. 7*B*) and confocal immunohistochemistry results (data not shown) showed no change in Sgk1 expression with Dex treatment, although another three sample sets of blots showed upregulation with Dex (Fig. 7*C*).

Glucocorticoids act at GR to regulate transcript expression. We investigated whether changes in transcript levels in response to Dex are via GR or MR. Dex-stimulated transcript expression of the α-subunit of ENaC and Sgk1 in SCCD was significantly inhibited only by the GR antagonist mifepristone (100 nM) but not by the MR antagonist spironolactone (10 μM) (Fig. 8), suggesting that Dex acts selectively at GR to exert changes in transcript expression levels.

Lack of effect of DMSO on the transcript expression. In our previous study (41) and in the present study, SCCD primary cultures were exposed to DMSO-dissolved chemical reagents along with water-dissolved Dex for about 8–24 h to understand the genomic stimulation of Na^+ transport by glucocorticoids. The final concentration of DMSO, used for long-term functional studies, never exceeded 0.1%. We investigated here whether DMSO (0.1% for 24 h) affected the transcript expression of α-ENaC, β_3-Na^+-K^+-ATPase, Kir7.1, and Sgk1 in SCCD monolayers. It was found that DMSO did not alter any of these transcript levels (Fig. 9). However, Dex (100 nM for 24 h) did alter the transcript levels (Figs. 2, 3, 4*E*, and 6), suggesting that the long-term functional changes shown in our previous study (41) and changes in transcript levels in the present study were specific effects and not affected by DMSO.

DISCUSSION

Our recent functional study (41) has shown that SCCD epithelia contribute to the low concentration of Na^+ in the vestibular endolymph under glucocorticoid control via GR. However, the transcript expression of cation transporters and key regulatory proteins involved in Na^+ transport and their regulation by glucocorticoids had not been investigated. The findings in our present study identify genes involved in amiloride-sensitive Na^+ transport pathway by SCCD epithelia and their regulation by glucocorticoids.

ENaC. The highly Na^+-selective ENaC, composed of α-, β-, and γ-subunits (1, 25), mediates Na^+ absorption in many epithelia with high electrical resistance (12, 47), such as SCCD epithelium. It has been found in mammals that the expression of the three ENaC subunits undergoes tissue-specific noncoordinated regulation depending on the physiological status (61), and this can occur at both the mRNA and protein levels.

Regulation of ENaC subunit expression by Dex in SCCD appears to be unusual among Na^+-transporting epithelia (61). Although glucocorticoids increase α-ENaC transcript expression in H441 as well as in SCCD, it is downregulated in fetal distal lung epithelial cells and does not change in A549 cells (61). Dex upregulation of only α-ENaC transcript expression in SCCD is consistent with the proposition that it is a limiting constituent that acts as a chaperone for the other subunits of ENaC in trafficking to the apical membrane (30). Even though we found an increased total protein expression for α- and γ-ENaC subunits, our results do not answer the question of whether apical membrane surface expression was altered with Dex because whole cell lysates but not membrane protein fractions were used to identify protein expression.

SUR1 has been implicated in the regulation of ENaC. It has been found in a *Xenopus laevis* oocyte expression system that rat SUR1, an ABC protein that shares a high degree of homology with the cystic fibrosis transmembrane conductance regulator (CFTR), inhibits rat ENaC activity by reducing the surface expression (24). It has also been demonstrated in mammalian expression systems that functional CFTR, which is thought to interact with ENaC (27), either inhibits ENaC

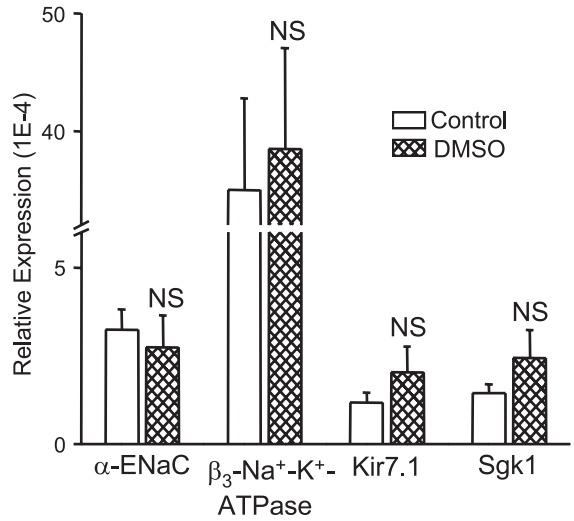

Fig. 9. Lack of effect of DMSO on transcript expression of α-ENaC, β_3-Na^+-K^+-ATPase, Kir7.1, and Sgk1. Real-time RT-PCR was performed on RNA obtained from primary cultures of SCCD epithelia before (control) and after incubation with 0.1% DMSO for 24 h. Values are expressed relative to 18S rRNA transcript expression (means ± SE); $n = 4$. Note the break in the vertical axis.

expression (54) or is required for ENaC activation (46). Expression of the transcripts for both SUR1 (Fig. 5*E*) and CFTR (unpublished observations) in SCCD suggests that ENaC may be regulated by SUR1 and CFTR in SCCD epithelium.

Na^+-K^+-ATPase. The functional Na^+ pump consists of an association of one α-catalytic subunit and one β-glycoprotein subunit of Na^+-K^+-ATPase. Some isoforms are expressed ubiquitously, whereas some are expressed in a tissue-specific manner (2, 39, 40, 52).

The transcript expression of more than one α- and β-subunit isoform indicates the possible diversity of Na^+ pumps in SCCD epithelium. The combinations formed of α_1- and α_3-isoforms with β_1- and β_3-isoforms is not known. Findings of Dex upregulation of the transcripts for β_2- and β_3-Na^+-K^+-ATPase isoforms suggests that the glycoprotein component (β-subunit) rather than the catalytic component (α-subunit) is important for glucocorticoid-stimulated Na^+ transport (41).

K^+ channels. Glucocorticoid-induced Na^+ transport by SCCD is sensitive to Ba^{2+} in the concentration range expected for inhibition of Kir K^+ channels. The strong rectifier Kir2.3 and the weak rectifier Kir7.1 (7) are the predominant Kir channel genes found to be expressed in the absence of Dex, whereas the strong rectifiers Kir2.1, Kir2.2, and Kir2.4 and the weak rectifier Kir7.1 (7) predominate in the presence of Dex. Upregulation of transcripts for Kir2.1, Kir2.2, Kir2.4, Kir4.2, and Kir7.1 by Dex is likely to reflect glucocorticoid-increased transepithelial Na^+ transport by SCCD epithelia via K^+ recycling across the basolateral membrane.

Kir3 subfamily channels are not likely functional in SCCD, although transcripts for Kir3.1 and Kir3.3 were found to be expressed and upregulated by Dex. Kir3.1 does not form functional homotetrameric channels (32), whereas the isoforms (Kir3.2 and Kir3.4) with which it forms functional heterotetramers (7) are not expressed in SCCD. Even though Kir3.1 can assemble with Kir3.3 (32), this combination only leads to reduced surface expression of Kir3.1, because Kir3.3 putatively targets Kir3.1 to lysosomes (32). Similar to Kir3.1, Kir5.1 is also a nonfunctional channel by itself (55). However, Kir5.1 can form functional channels by assembling with either Kir4.1 or Kir4.2 (55), and all three isoforms are expressed in SCCD. Absence of the transcripts for both Kir6.1 and Kir6.2 and SUR2 in SCCD epithelia is in agreement with the absence of Glib-inhibitable I_{sc} after Dex stimulation.

Regulation of ENaC and Na^+-K^+-ATPase via Sgk1 and Nedd4-2. Three isoforms of Sgk (Sgk1, Sgk2, and Sgk3) and two isoforms of Nedd4 (Nedd4-1 and Nedd4-2) have been identified in mammalian tissues (19, 23). Even though all isoforms of both Sgk and Nedd4 have been suggested to be potential regulators of ENaC in expression systems (14, 19), it is likely that Sgk1 and Nedd4-2 are physiological regulators of ENaC. Interestingly, only the transcript of Sgk1, but not Sgk2 and Sgk3, was suggested to be regulated by glucocorticoids (23).

Nedd4-2 is a ubiquitin protein ligase that binds to and ubiquitinates ENaC subunits (51, 53). Ubiquitinated ENaC then undergoes endocytosis from the apical membrane and subsequent proteasomal degradation (51, 53). Na^+ absorption is thereby decreased by Nedd4-2 by reducing the expression of ENaC channels in the apical membrane. Sgk1 binds to and inactivates Nedd4-2 by phosphorylation, which prevents binding and ubiquitinization of ENaC. The longer residency time for ENaC in the apical membrane leads to increased Na^+ absorption (10, 51).

Transcripts for both Sgk1 and Nedd4-2 in SCCD were upregulated by Dex, despite their contrasting effects on ENaC surface expression levels and on Na^+ absorption. Nevertheless, transcript expression for the positive regulatory protein Sgk1 was increased by about twice that of the negative regulatory protein Nedd4-2. This mRNA expression profile is consistent with Dex-stimulated I_{sc} measurements, although there may not be a one-to-one correspondence between mRNA levels and protein expression levels. The apparent discrepancy between the Nedd4-2 transcript upregulation and I_{sc} increase suggests the presence of other Dex-induced signaling pathways involved with Nedd4-2 in SCCD epithelium.

Rapid changes in Sgk1 expression have been observed in other ENaC-mediated Na^+ transport systems. It has been found in model Na^+-transporting epithelial cells, H441 cells (21), and A6 cells (6) that the expression of Sgk1 transcripts after glucocorticoid stimulation reached a peak at about 1 h, declined over the next 24 h, and remained higher than the basal level at 24 h (6, 21). Furthermore, Sgk1 protein expression in A6 cells increased significantly at about 30 min after glucocorticoid stimulation, increased further for about 6 h, and finally declined to a nearly basal or slightly elevated level at 24 h, although the authors did not comment on this result (6). Despite little change in Sgk1 protein expression, it was found in A6 cells that Dex increased Na^+ currents at 24 h about threefold and that this was due to increased channel density rather than open probability (16). Similarly, Dex stimulated amiloride-sensitive I_{sc} in H441 cells expressing ENaC by 10- to 20-fold (45). These data are consistent with our findings reported here at 24 h. Our variable results with Sgk1 protein expression suggest that increased signaling from Sgk1 does not necessarily depend on a maintained elevation of the Sgk1 protein level.

A very recent report (1a) has suggested that Sgk1 expression increases Na^+-K^+-ATPase activity in A6 renal epithelial cells independent of changes in protein expression or abundance in the plasma membrane, even though the exact mechanism of activation is not understood yet. On the other hand, a few reports (56, 63) have suggested that Sgk1 increases Na^+-K^+-ATPase surface expression and activity in *Xenopus laevis* oocytes by translocating cytoplasmic Na^+-K^+-ATPase pumps to the plasma membrane.

Regulation of receptors and agonists. Genomic stimulation of Na^+ transport in target tissues by glucocorticoids is linked not only to the activation of intracellular corticosteroid receptors but also to the expression of functional intracellular 11β-HSD isoforms. 11β-HSDs are enzymes that catalyze the interconversion of active and inactive forms of the agonists. The concentration of agonists in corticosteroid-responsive target tissues is thereby determined by not only plasma glucocorticoid hormone levels but also intracellular 11β-HSD isoforms (22).

11β-HSD1 catalyzes the conversion of inactive cortisone (humans and most mammals) and 11-dehydrocorticosterone (rats and mice) to active cortisol (humans and most mammals) and corticosterone (rats and mice), respectively (49). Therefore, 11β-HSD1 increases the concentration of active intracellular glucocorticoids. 11β-HSD2 catalyzes the conversion of active cortisol and corticosterone to inert cortisone and 11-

dehydrocorticosterone (13) and protects MR from promiscuous binding of glucocorticoids. Therefore, 11β-HSD2 confers specificity to the mineralocorticoid aldosterone (35).

It has been suggested that 11β-HSD1 is found in a wide range of tissues (5), especially in glucocorticoid-selective responsive tissues such as the liver (22, 49), adipose tissue (43), and epithelia of the proximal nephron (48), whereas 11β-HSD2 is found mainly in mineralocorticoid-selective target tissues such as epithelia of the distal nephron and colon (5, 13, 49). One group (48) has demonstrated that transcripts for 11β-HSD2 and MR are highly expressed in the distal nephron, whereas transcripts for 11β-HSD1 and GR are highly expressed in the proximal nephron.

The amount of the transcript in SCCD for GR was found to be ~25 and ~6 times higher than that of MR in the absence and presence of Dex, respectively. Similarly, the transcript for 11β-HSD1 was found to be approximately four times higher than that of 11β-HSD2 in the absence of Dex. Predominant expression of GR and 11β-HSD1 mRNA compared with MR and 11β-HSD2 in SCCD epithelia is consistent with SCCD being specifically responsive to glucocorticoids.

Three cytosolic (α, β, and γ) and a membrane-bound isoforms of GR have been identified in human tissues and cells (4, 17, 42). Only α-GR has been found to bind to hormones and to transactivate target genes (42). β-GR expression has been found to be absent in mouse tissues, a conclusion that was extended to the rat (38). To our knowledge, only one GR has been identified in rats, and our primers were designed to amplify rat GR.

Downregulation of the transcripts for both GR and 11β-HSD1 by Dex suggests negative feedback of prolonged exposure to a high level of agonist. This local feedback mechanism is likely a means to protect cells against excessive and chronic hormone action (11, 20, 22, 28, 58).

In conclusion, the lumen of semicircular canals of the vestibular system is filled with endolymph, a fluid with a high concentration of K^+ (149 mM) and a low concentration of Na^+ (9 mM) (60). This uncommon extracellular composition is required to support transduction of head acceleration into nerve impulses in the vestibular system. Our current findings of Dex regulation of the transcript expression of key genes involved in Na^+ transport are consistent with a previous functional study (41) of genomic stimulation by physiological and therapeutic glucocorticoids of GR-dependent Na^+ transport by SCCD epithelia to maintain low levels of Na^+ in the vestibular endolymph. The present findings provide the basis to understand at the transcriptional level the glucocorticoid-stimulated increase in Na^+ absorption by the SCCD.

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