Glucocorticoid Induction of Epithelial Sodium Channel Expression in Lung and Renal Epithelia Occurs via trans-Activation of a Hormone Response Element in the 5′-Flanking Region of the Human Epithelial Sodium Channel α Subunit Gene*

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From the 1Department of Internal Medicine, University of Iowa College of Medicine and the Veterans Affairs Medical Center, Iowa City, Iowa 52246

In airway and renal epithelia, the glucocorticoid-mediated stimulation of amiloride-sensitive Na⁺ transport is associated with increased expression of the epithelial Na⁺ channel α subunit (αENaC). In H441 lung cells, 100 nM dexamethasone increases amiloride-sensitive short-circuit current (3.3 μA/cm² to 7.5 μA/cm²), correlating with a 5-fold increase in αENaC mRNA expression that could be blocked by actinomycin D. To explore transcriptional regulation of αENaC, the human αENaC 5′-flanking region was cloned and tested in H441 cells. By deletion analysis, a ~150-base pair region 5′ to the upstream promoter was identified that, when stimulated with 100 nM dexamethasone, increased luciferase expression 15-fold. This region, which contains two imperfect GREs, also functioned when coupled to a heterologous promoter. When individually tested, only the downstream GRE functioned in cis and bound GR in a gel mobility shift assay. In the M-1 collecting duct line Na⁺ transport, moENaC expression and luciferase expression from αENaC genomic fragments were also increased by 100 nM dexamethasone. In a colonic cell line, HT29, trans-activation via a heterologously expressed glucocorticoid receptor restored glucocorticoid-stimulated αENaC gene transcription. We conclude that glucocorticoids stimulate αENaC expression in kidney and lung via activation of a hormone response element in the 5′-flanking region of hoENaC and this response, in part, is the likely basis for the up-regulation of Na⁺ transport in these sites.

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‡ These authors contributed equally to this work.

§ To whom correspondence should be addressed: Div. of Nephrology, Dept. of Internal Medicine, University of Iowa Hospitals and Clinics, 200 Hawkins Dr., Iowa City, IA 52242-1081. Tel.: 319-356-4216; Fax: 319-356-2999; E-mail: christie-thomas@uiowa.edu.

1 The abbreviations used are: ENaC, epithelial sodium channel; MC, mineralocorticoid(s); GC, glucocorticoid(s); IMCD, inner medullary collecting duct; Isc, short circuit current; Rb⁺, resistance; RPA, ribonuclease protection assay; bp, base pair(s); nt, nucleotides; UTR, untranslated region; GRE, glucocorticoid response element; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; CCD, cortical collecting duct; PNMT, phenylethanolamine N-methyltransferase; SRC-1, steroid receptor co-activator 1; SMRT, silencing mediator for retinoid and thyroid hormone receptors; PAGE, polyacrylamide gel electrophoresis.

The mechanism of the increase in steady state levels of ENaC mRNAs following adrenal steroid treatment is unknown but has been presumed to be at the level of ENaC gene transcription.

In this study, we identify the promoter and enhancer elements in the 5′-flanking region of the human αENaC gene that regulate basal and GC-mediated induction of αENaC gene expression. In addition we provide evidence that enhanced Na⁺ transport by two different cell lines (lung and kidney) is tightly linked to enhanced expression of αENaC.

EXPERIMENTAL PROCEDURES

Cycloheximide, dexamethasone, spironolactone, amiloride, and human placental collagen were purchased from Sigma. Actinomycin D was obtained from Roche Molecular Biochemicals, poly dI-dC from Amer...
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sham Pharmacia Biotech, and RU38486 was a generous gift from Roussel Uclaf (Romainville, France). Culture materials were from Life Technologies, Inc., and all radionucleotides were from NEN Life Science Products. Stock solutions of cycloheximide and actinomycin D were made in MeSO and stocks of dexamethasone, spironolactone, and RU38486 made in ethanol.

Tissue Culture and RNA Isolation—The human lung epithelial cell line, H441, and the human colon carcinoma cell line, HT29, were cultured as described previously (16). The mouse renal cortical collecting duct (CCD) cell line, M-1, was grown in Dulbecco’s modified Eagle’s medium:F12 with 10% fetal calf serum (17). To examine the effects of dexamethasone on gene expression, cell cultures were switched to serum-free medium and then exposed to various concentrations of dexamethasone or its vehicle for 24 h. Cycloheximide (10 μM), actinomycin D (1 μM), and the steroid receptor blockers, spironolactone (10 μM) and RU38486 (10 μM), were used in some experiments and compared with control cultures in the presence of vehicle alone. RNA was isolated from these cells as described previously (16).

Na⁺ Transport Measurements—H441 and M-1 cells were seeded on 12-mm Millicell PCF filters (Millipore, Bedford, MA), which had been treated with human placental collagen. H441 cells were grown for 6 days in RPMI medium with 6% serum and 100 nM dexamethasone and the medium changed daily. A day prior to electrical measurements, the cells were placed in steroid-free medium and the following day placed in serum-free medium (or without 100 nM dexamethasone). The H441 filters were then transferred to a specially designed chamber (Jim’s Instruments, Iowa City, IA) to measure transepithelial voltage, resistance (Rₑ), and short circuit current (Iₑ) at 37°C (18). M-1 cells were grown for 3 days in Dulbecco’s modified Eagle’s medium:F12 supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 mM triiodothyronine, 50 nM hydrocortisone, 10 mM sodium selenite, 50 μg/ml gentamicin, 10 μg/ml bovine serum albumin, and 5 mM dexamethasone. The filters were then grown for 4 days without albumin and steroids and then with or without 100 nM dexamethasone and 10 μM spironolactone for another 24 h.

Ribonuclease Protection Assay (RPA)—To measure steady state levels of αENaC gene expression, a previously described cDNA template that would distinguish exon 1A-initiated (αENaC-1) from exon 1B-initiated (αENaC-2) (3, 4) transcripts was used to synthesize antisense [α-32P]cRNA (16). To control for RNA extraction, quantitation and gel loading, an 18 S rRNA template (pTR1–18SRNA, Ambion, Austin, TX) was used. Sample RNAs were co-hybridized overnight with ENaC and 18 S riboprobes, digested with RNase A and T1, and analyzed by PAGE as described previously (16).

To quantitate mRNA expression, the autoradiograms were scanned with a PDI scanning densitometer and the density of individual bands measured using Quantity One software (Huntington Station, NY). Each aliquot of cells was then diluted into 2 ml of a solution containing 120 mM KCl, 150 mM CaCl₂, 10 mM K₂HPO₄, 10 mM KH₂PO₄, 2 mM EGTA 5 mM MgCl₂, 100 mM ATP, 250 mM glutathione, and 25 mM Hepes, pH 7.6. A 500-μl aliquot of cells was then diluted with 20 μg of a luciferase construct and 40 μg of pSVβ-gal, where the Escherichia coli lucZ gene is cloned downstream of the SV40 promoter (Promega) was combined with LipofectAMINE (Life Technologies, Inc.) and added to each well. For transfection of M-1 cells, monolayers from two T75 flasks were trypsinized, and then resuspended in 2 ml of a solution containing 120 mM KCl, 150 mM CaCl₂, 10 mM K₂HPO₄, 10 mM KH₂PO₄, 2 mM EGTA 5 mM MgCl₂, 100 mM ATP, 250 mM glutathione, and 25 mM Hepes, pH 7.6. A 500-μl aliquot of cells was then diluted with 20 μg of a luciferase construct and 40 μg of pSVβ-gal in a 0.4-cm cuvette and then electroporated (Electroporator II, Invitrogen, Carlsbad, CA) at 330 V, 1000 microfarads, and 500 ohms. Each aliquot of cells was then diluted with complete medium and plated into four to six wells of a 12-well plate. 24–48 h following transfection, cells were placed in serum-free medium and dexamethasone or vehicle were added where appropriate; another 24 h later, cell lysates were prepared for measurement of reporter gene activity. For HT29 cells, LipofectAMINE Plus (Life Technologies, Inc.) was used as the transfecting reagent. As HT29 cells contained a high level of endogenous β-galactosidase activity, we used pRL-SV40 (Promega), where the sea pansy (Renilla reniformis) luciferase gene is cloned downstream of the SV40 promoter, as the internal control plasmid. In some experiments, 1 μg of the plasmid p6RGR, where the rat glucocorticoid receptor cDNA is under the control of the Rous sarcoma virus promoter (gift from D. Pierce and K. Yamamoto) was co-transfected with the luciferase vectors.

For preparation of cell lysates, the cells were washed in phosphate-buffered saline and then scraped into Lysis buffer (Luciferase assay kit, Promega). An aliquot of cell lysate was added to Luciferase Assay reagent and activity measured in a Monolight 2010 luminometer (Analytical Luminescence Laboratories, Ann Arbor, MI) for 5 s. For measurement of β-galactosidase activity, an equal aliquot of cell lysate was incubated with the substrate Galacto-Plus (Tropix, Bedford, MA) for...
30 to 60 min and then activity measured in a luminometer for 5 s. Lysates from HT29 cells were made with Passive Lysis Buffer (Promega) and firefly luciferase activity, reflecting transcriptional strength of cloned ENaC gene fragments, and sea pansy luciferase activity was sequentially measured in the same sample using the Dual Luciferase Reporter assay kit (Promega).

**Gel Mobility Shift Assay—**Oligonucleotides that correspond to the functional ENaC GRE or to a nonspecific sequence were synthesized and annealed together (see sequences below).

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\text{Dn-GRE: } \text{gatcCACTAGTAGAACAGAATGTCCTAG} \\
\text{Nonspecific (NS) Seq: gatcCGGCAGCTGTGCAAATCCTG} \\
\text{GCCGTCGACACGTTAGGAC} \\
\text{gtacCTAGTAGAAGAATGTCCTAG} \\
\text{GTGATCATCTTGTCTTACAGGATC} \\
\text{gtagCAGCTGCCGAAAATCCTG} \\
\text{GCCGTCGACACGTTAGGAC} \\
\text{GTGATCATCTTGTCTTACAGGATC} \\
\text{gtacCTAGTAGAAGAATGTCCTAG} \\
\text{GTGATCATCTTGTCTTACAGGATC} \\
\text{gtagCAGCTGCCGAAAATCCTG} \\
\text{ GCCGTCGACACGTTAGGAC}
\]

**Sequences 4 and 5**

For gel mobility shift experiments, 50,000 cpm of end-labeled double-stranded oligonucleotides were incubated with recombinant human glucocorticoid receptor (Affinity Bioreagents Inc., Golden, CO) in a 20-μl reaction mixture that contained 20 mM Hepes, pH 7.9, 60 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 10 ng/ml poly(dI-dC), 5 μg/ml bovine serum albumin, and 10% glycerol. For competition experiments, a 50-fold excess of cold oligonucleotides were used. All constituents except the labeled probe were preincubated at 4 °C for 30 min and then incubated with the labeled probe at 22 °C for 30 min. Samples were then resolved on a 3.5% nondenaturing polyacrylamide gel (acrylamide: bisacrylamide 20:1) in 0.5× TBE buffer run at 150 V.

**RESULTS**

GC increases αENaC mRNA expression in lung and kidney cortex and in cultured epithelial cells derived from these tissues (8–11, 15). To address the mechanisms whereby GC increase αENaC mRNA expression, we used a human lung cell line H441 and mouse CCD cell line M-1. We found that H441 cells, when grown on permeable supports in the presence of dexamethasone, develop as a tight epithelium and have electrogenic ion transport measured as a positive Isc from the apical to the basolateral compartment (Fig. 1A). A current was almost completely blocked by 10 μM amiloride applied to the apical side (Fig. 1B), indicating that the bulk of the Isc was accounted for by amiloride-sensitive electrogenic Na⁺ transport pathways. To examine the effect of dexamethasone on Na⁺ transport, H441 cells were placed in steroid-free media for 24 h and then exposed to 100 nM dexamethasone or vehicle for another 24 h. Dexamethasone increased Isc from 3.3 μA/cm² to 7.5 μA/cm² (Fig. 1C), similar to results obtained from primary cultures of airway epithelial cells (15).

We have previously reported that heterogeneity in αENaC transcripts arise from alternate transcription start sites and from splicing at the 5’ end of hoENaC (16). exon 1A begins at the upstream transcription start site and gives rise to αENaC-1 while a second transcription start site 724 bp downstream in an alternate first exon (exon 1B) gives rise to αENaC-2,3,4 (Fig. 2). 24 h of treatment with dexamethasone increased expression of both αENaC transcripts in a dose-dependent manner with the earliest effect seen at 10 nM (Fig. 3A). The basal expression of exon 1A-initiated transcripts was greater than exon 1B-initiated transcripts, and this difference persisted through the dose-response curve. The effect of GC on αENaC expression was blocked by RU38486, a type II (glucocorticoid receptor; GR) antagonist, but not by spironolactone, a type I (mineralocorticoid receptor; MR) antagonist, confirming that these effects required GR binding (Fig. 3B). The effect of GC on αENaC expression was blocked by co-administration of actinomycin D, an inhibitor of transcription, suggesting that GC stimulates αENaC expression by increasing gene transcription rather than by affecting mRNA stability (Fig. 3C). Cycloheximide, a
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Fig. 4. A, separate promoters drive expression of transcripts αENaC-1 and αENaC-2 and a dexamethasone-responsive region maps to a site between −487 and −142 of the human αENaC gene. Left panel, 5′ end of αENaC including the 5′-flanking region showing putative transcription factor binding motifs. The location and extent of genomic fragments coupled to the luciferase (luc) coding region are shown. The αENaC transcription start sites are shown as bent arrows. Right panel, the genomic constructs were compared with the empty plasmid pGL3basic and the luciferase assay corrected for β-gal activity is shown. Each construct was tested with (black bar) and without (white bar) dexamethasone (100 nM for 24 h). *, p < 0.002 compared with pGL3basic; #, p < 0.02 compared with the absence of dexamethasone; +, p < 0.002 compared with the absence of dexamethasone. (n = 3–4 determinations). Panels B and C, mapping of promoters P1 and P2. B, the minimal sequence for transcriptional activity of P1 is contained within the −82+44 fragment. *, p < 0.001 compared with pGL3basic; #, p < 0.001 compared with −99+44 construct. C, the minimal sequence for transcriptional activity of P2 is contained within the +476+814 fragment. *, p < 0.002 compared with pGL3basic.

To examine the mechanism of transcriptional regulation of the αENaC gene, we cloned 5′-flanking sequences upstream of both transcription start sites and evaluated these by transient transfection assays. We focused our attention on identification of the putative promoters as well as the GC-regulated elements of the αENaC gene. Constructs that included either transcription start site and portions of their proximal 5′-flanking sequence were able to stimulate luciferase expression in H441 cells (Fig. 4A). The ability of constructs containing sequence 5′ to each transcription start site (e.g., −142 +44 and +322 +814) to increase luciferase gene transcription suggest that separate promoters direct expression of αENaC-1 and αENaC-2. Two of the five tested constructs robustly stimulated luciferase activity when treated with 100 nm dexamethasone (Fig. 4A). Analysis of the sequence common to these constructs (−487 and −142) revealed that it contained one or more GREs. The magnitude of the dexamethasone-induced luciferase gene transcription is similar to the magnitude of αENaC mRNA stimulation in H441 cells (Fig. 3A), suggesting that transcriptional activation of this region is sufficient to account for the effect of dexamethasone on steady state αENaC mRNA levels. A preliminary analysis of the nucleotide sequence in this region between −289 and −142 showed two imperfect GREs, TGTcCANN (Up-GRE) and AGAACAnn (Dn-GRE), which are candidate cis-elements to mediate this effect.

To define the promoter regions P1 and P2, further deletions of the constructs −142+44 and +322+814, respectively, were made and tested. In the case of the P1 promoter, a construct as short as −82 to +44 was active, suggesting that the minimal promoter included within this region (Fig. 4B). In the case of the P2 promoter, a construct as short as +476 to +814 was active, suggesting that the minimal promoter included sequences 5′ to this construct (Fig. 4C). To determine if the region between −289 and −143 in the 5′-flanking region of the αENaC gene could transduce the glucocorticoid effect, a 146-bp sequence containing these elements were cloned in reverse orientation (−289−143Inv) upstream of the P1 and P2 promoters and transfected into H441 cells. As expected for a classic “enhancer,” these elements functioned in reverse orientation to confer glucocorticoid responsiveness to both promoters (Fig. 5, A and B). These experiments confirm that the defined region is sufficient to direct glucocor-
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To further define transcriptional regulatory elements necessary for GC induction, a new set of 5′ deletion constructs were made and tested in H441 cells (Fig. 6, A and B). To determine the role of each GRE, single copies of double-stranded oligonucleotides corresponding to each 15-nucleotide region were cloned upstream of the αENaC-1 promoter and tested in H441 cells (Fig. 6B). These results confirmed that the region containing the Dn-GRE (−248 to −142) was sufficient to confer GC enhancement. First, the −248 to −44 construct, which excludes the Up-GRE, is capable of responding to dexamethasone (Fig. 6A); inclusion of the Up-GRE sequence (−389 to −44) does not alter either basal or the GC-enhanced response (Fig. 6B). Second, the construct that contains only the Dn-GRE permits GC enhancement, but the construct containing only the Up-GRE does not (Fig. 6B). Finally, a 4-nt mutation that is predicted to disrupt GR binding abolished GC-mediated luciferase expression from the Dn-GRE (Fig. 6D). The region immediately 5′ to the Dn-GRE also appeared to contain an inhibitory element as constitutive expression of the −248 to −142 construct was lower than the −142 to +44 construct (Fig. 6A).

To determine if the identified Dn-GRE could bind GR in a mobility shift DNA binding assay, oligonucleotides corresponding to these sequences were synthesized, incubated with purified GR, and then analyzed by PAGE and autoradiography. To test specificity of interaction, the purified GR was preincubated with an excess of cold oligonucleotides corresponding to Dn-GRE, Up-GRE, or a nonspecific oligonucleotide prior to incubation with labeled oligonucleotide. Our results indicate that GR binds specifically to the functional Dn-GRE to retard its mobility (Fig. 7).

We next examined amiloride-sensitive Na+ transport and αENaC gene expression in M-1 cells, a mouse CCD cell line. This cell line, established from an SV40 transgenic mouse, appears to have many of the characteristics of the native CCD including the presence of amiloride-sensitive Na+ transport (17). Dexamethasone treatment leads to marked stimulation of Na+ transport within 24 h (Fig. 8A). To determine the mechanism of this effect, we examined the expression of mouse αENaC mRNA in these cells when treated with dexamethasone. We show a potent stimulation of αENaC mRNA expression by 100 nM dexamethasone in these cells (Fig. 8B). To determine if the stimulation of αENaC mRNA expression occurs at the level of gene transcription, an αENaC-luciferase construct that included the GC-responsive region (−487 to +661) was transfected into M-1 cells and then the effect of 24 h of stimulation with 100 nM dexamethasone was studied. As in H441 cells, dexamethasone increased luciferase gene transcription via αENaC genomic fragments about 5–10-fold (Fig. 8C). These experiments suggest that the GC-mediated induction of αENaC gene transcription is similar in airway epithelia and in CCD.

To confirm that the GR is required for the GC effect on αENaC gene transcription, we studied HT29 cells, a human colonic epithelial cell line. While these cells express the αENaC mRNA constitutively (16), we had previously noted that there was no detectable increase in mRNA expression with GC.2 When the αENaC gene construct containing the GC-responsive regions (−487 to +661) was transfected into these cells, constitutive or basal activation was seen consistent with the presence of an active αENaC promoter (Fig. 8D). When treated with 100 nM dexamethasone, no further increase was seen unless the GR was co-transfected with the luciferase construct (Fig. 8D). These results suggest that these cells lack GR or that the endogenous GR is not available for binding or trans-activation following stimulation with GC. Complementation with heterologously expressed receptor confirms that the GR is required for the GC effect on αENaC gene transcription.

DISCUSSION

Corticosteroids are important physiological regulators of transepithelial sodium transport in the distal nephron, the distal colon, and the airway epithelia. With the cloning of the ENaC subunits, the effect of MC and GC on ENaC mRNA expression has been studied in a variety of epithelial cells and tissues from many species. The chronic effects of these hormones on Na+ transport coincide with an increase in mRNA expression of one or more subunits of the ENaC complex suggesting that synthesis of new ENaC channels may account for the increase in Na+ transport. However, the exact mechanism of the corticosteroid regulation of ENaC subunits is unknown.

We examined αENaC expression in H441 cells, a human lung cell line where all ENaC subunits are expressed (α, γ (Ref. 16 and 19); β (see Footnote 2)). When allowed to grow as a polarized epithelium, these cells develop an amiloride-sensitive Isc that is enhanced by GC treatment (Fig. 1). We then used these cells to explore the effect of GC on αENaC gene expression. It is important to note that this cell line expresses all four principal transcripts of αENaC described to date and the transcription start sites are identical to that seen in human

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2 C. P. Thomas, unpublished observation.
kidney and lung tissue (16). We show that GC increased expression of both exon 1A- and 1B-initiated transcripts in a dose-dependent manner and that this effect was blocked by RU38486 and by actinomycin D. These results suggested that gene transcription following receptor binding is required for the GC effect on ENaC expression in these cells.

To address the mechanisms of transcriptional regulation of the ENaC gene and to identify its promoters, we cloned portions of the 5'-flanking region. Sequence analysis of the region upstream of each transcription start site did not reveal a canonical TATA box. TATA-less promoters tend to initiate transcription at multiple sites. Thus, it seemed possible that a single upstream promoter could initiate transcription at both ENaC start sites. Our results show that genomic fragments upstream of each transcription start site did not reveal the presence or absence of cold competitor and then analyzed by PAGE. GR binds to Dn-GRE to retard its mobility in lanes 2 and 3 ("shifted probe"). The binding of GR is inhibited strongly by a 50-fold excess of cold Dn-GRE (lane 4), and weakly by an excess of cold Up-GRE (lane 5) but not by a nonspecific (NS) competitor (lane 6).
**FIG. 8.** Glucocorticoid-mediated αENaC gene transcription in M-1 and HT29 cells. Panel A, amiloride-sensitive Isc in M-1 cells treated for 24 h with dexamethasone (dex). Dexamethasone increases Isc in M-1 cells (n = 15–16 determinations ± S.E.). *, p < 0.001 compared with control. Panel B, αENaC and β-actin mRNA expression following dexamethasone stimulation for 24 h in M-1 cells. Dexamethasone increases αENaC mRNA expression (measured by RPA and expressed as a ratio of β-actin levels) in M-1 cells (n = 3 ± S.E.1, *, p < 0.02 compared with control. Panel C, the human αENaC construct −αENaC−487 + 661 or pGL3basic was transfected into M-1 cells and then exposed to dexamethasone and luciferase activity measured 24 h later. Dexamethasone stimulates transcription of an αENaC/luciferase chimeric construct. *, p < 0.001 compared with pGL3basic; #, p < 0.01 compared with control. Panel D, the human αENaC construct −αENaC−487 + 661 co-transfected with the rat GR in HT29 cells, then exposed to dexamethasone and luciferase activity measured 24 h later. The αENaC construct shows constitutive activity but no further stimulation with dexamethasone unless the GR is exogenously expressed, p < 0.05 compared with control.

adjacent to the Dn-GRE (between −248 and −142) are required for the full glucocorticoid effect as the Dn-GRE alone increased gene transcription by just 3-fold compared with 15-fold with the −248+44 construct (Fig. 6B).

We have demonstrated by transfection, gel mobility shift, and trans-activation assays that glucocorticoids acting through their cognate receptor stimulate αENaC gene transcription via an imperfect GRE upstream of the hαENaC gene. GREs can bind MR in vitro, and so far no distinct mineralocorticoid response elements have been identified (24). Currently, the GREs are thought to be the natural targets for activated MR. In vivo, in aldosterone-responsive epithelia the effect of GC on ENaC expression is very similar to the effect of MC (1) and it is quite likely that the MC effect is also via the αENaC GRE. It is important to note, however, that the molecular responses to MC and GC have some differences (12, 25). Mechanisms proposed to explain these differences include the specific metabolism of endogenous GC to an inactive metabolite in aldosterone-responsive epithelia and the combinatorial regulation by GR but not MR at composite response elements for steroid receptors and other trans-acting factors (26, 27).

The effect of corticosteroids on ENaC gene expression in vivo is tissue-specific with distinct effects on different subunits in the kidney, colon, and lung. For example, dexamethasone and aldosterone increase αENaC mRNA in the kidney, but β and γENaC mRNA in colon (8–10, 28). Under certain culture conditions, we can induce αENaC gene transcription in response to glucocorticoids in a colonic cell line, HT29 (Fig. 8D). The fact that the αENaC gene is not induced in the colon in vivo even though steroid receptors are present implies that the effect of corticosteroids on these subunits is not a simple consequence of receptor binding and activation of a GRE. The factors that determine tissue-specific regulation may include steroid receptor cofactors such as SRC-1 and SMRT (29, 30) but this remains to be elucidated.

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