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# Glucocorticoid receptor is indispensable for physiological responses to aldosterone in epithelial $Na^+$ channel induction via the mineralocorticoid receptor in a human colonic cell line

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ABSTRACT

The epithelial Na+ channel (ENaC) plays a crucial role in electrogenic $Na^+$ absorption in the distal colon. ENaC induction via the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) is differentially regulated by modulatory components. As most existing epithelial cell lines including colonic epithelial cell lines miss the co-expression of functional GR and MR, signaling on ENaC is only poorly characterized regarding the interplay of glucocorticoids and mineralocorticoids.

In the present study, we show that GR expression and activity are indispensable for MR-dependent induction of ENaC-mediated $Na^+$ transport. Cooperativity of the two receptors has been studied in the highly differentiated, epithelial colonic cell line HT-29/B6-GR/MR which is equipped with the complete receptor repertoire of both GR and MR due to stable transfection. In contrast to HT-29/B6 cells solely expressing the MR, this cell line displays a physiological response to aldosterone regarding ENaC induction. To achieve this, a pre-incubation step with the GR agonist dexamethasone was required to allow for the subsequent stimulation of ENaC by aldosterone. As a result of cooperative effects between the activated GR and the MR, MR protein levels were elevated and MR-dependent transcription of ENaC subunits β and γ was increased. As an additional mechanism involved, transcription of SGK-1 (serum- and glucocorticoid-induced kinase 1) and GILZ (glucocorticoid-induced leucin zipper) – both essential for the insertion of ENaC into the apical enterocyte membrane – were also augmented by the activated MR.


## Introduction

The limiting factor for electrogenic $Na^+$ transport in the distal colon and other mineralocorticoid-sensitive, salt-absorbing epithelia is the epithelial $Na^+$ channel (ENaC) (Canessa et al., 1994). ENaC is composed of three subunits (α, β and γ), all of which are necessary for ENaC's efficient targeting to the apical cell surface and the promotion of significant $Na^+$ transport rates (Firsov et al., 1998). In the distal colon, glucocorticoid and mineralocorticoid hormones induce transcription of β- and γ-ENaC, whereas expression of α-ENaC is constitutive and remains unchanged (Epple et al., 2000). Induction of both β- and γ-ENaC is dependent on transactivation by the activated glucocorticoid (GR) or mineralocorticoid receptors (MR). A shared hormone response element (HRE) for this induction is postulated distant from the proximal promoter region of the two genes (Thomas et al., 2002). Furthermore, ENaC activity is influenced by corticosteroids on the post-translational level as several GR- and MR-dependent genes have been identified which reduce channel ubiquitination and thus internalization from the apical membrane (Verrey et al., 2008). Thereby, channel density on the apical surface of epithelial cells can be enhanced (Butterworth et al., 2009).

Hitherto, discrimination of the regulatory impact on ENaC by GR and MR in the GI tract has been hampered due to the absence of a colonic cell model co-expressing both functional GR and MR (Mick et al., 2001; Zeissig et al., 2006). Differential effects of these two receptors have already been revealed in chronic intestinal inflammation. For example, ENaC induction by the synthetic glucocorticoid dexamethasone is synergistically upregulated by the pro-inflammatory cytokine TNF-α (tumor necrosis factor-α), but increase in ENaC activity by the mineralocorticoid aldosterone via MR is impaired by TNF-α (Bergann et al., 2009a; Amasheh et al., 2004; Zeissig et al., 2008). However, molecular mechanisms underlying these observations have only been poorly characterized so far.

As has been previously shown by our group, colonic epithelial cells expressing the MR without co-expression of the GR exhibit

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functional ENaC in response to MR-specific doses of aldosterone only in the additional presence of the short-chain fatty acid butyrate which acts as a histone deacetylase inhibitor (Bergann et al., 2009b). In the present study, we sought to investigate a cell model highly resembling native colonic epithelial cells with respect to the set of expressed corticosteroid receptors which include both GR and MR (Schoneveld et al., 2004; Viengchareun et al., 2007). Thus, we introduce here a novel colonic cell model co-expressing functional GR and MR (HT-29/B6-GR/MR) which display functional ENaC due to mineralocorticoid incubation exclusively after the pre-stimulation of the GR with dexamethasone.

## Materials and methods

### *Cell culture and reagents*

HT-29/B6 cells represent a subclone of the human colorectal cancer cell line HT-29 growing as highly differentiated polarized epithelial monolayers (Kreusel et al., 1991). Clones stably expressing the human glucocorticoid receptor α (HT-29/B6-GR) or human mineralocorticoid receptor (HT-29/B6-Tet-On-MR) – abbreviated here as HT-29/B6-MR – were obtained by stable transfection as described earlier (Bergann et al., 2009b; Zeissig et al., 2006). HT-29/B6-GR/MR cells were cultivated in the presence of G418 (300 μg/ml) and hygromycin B (200 μg/ml; both PAA, Pasching, Austria). Prior to experimental use, cells were cultivated with hormone-free medium for 24 h. For dexamethasone pre-incubation, cells were grown on flasks for 7 days in the presence of dexamethasone (50 nM), then seeded onto permeable supports. After another 7 days of culture with dexamethasone, confluent cells were incubated with hormone-free medium for 24 h prior to the experiment. For experimental use, cells were seeded on Millicell PCF filters (3 μm) (Millipore, Schwalbach, Germany). Experiments were performed on day 7 when polarized monolayers reached confluence, giving transepithelial resistances ($R_t$) of around 600 Ω cm$^2$. 24 h prior to the experiment, cells were cultivated in medium with 10% hormone-free FCS (Biochrom AG, Berlin, Germany). RU 486 (10 and 5 μM; Sigma–Aldrich) was used as the GR antagonist. As MR antagonists, 1 μM spironolactone and 1 μM RU 26752 (both from Sigma–Aldrich, Steinheim, Germany) were applied. If not stated otherwise, aldosterone (Sigma–Aldrich) and sodium butyrate (Merck, Berlin, Germany) were used in a concentration of 3 nM and 2 or 5 mM, respectively.

### *Stable transfection of HT-29/B6-GR cells with human mineralocorticoid receptor*

The human hMR coding sequence was amplified and inserted into the pcDNA 3.1(+) (Invitrogen, Karlsruhe, Germany) expression vector containing a Zeocin selection marker as earlier described (Bergann et al., 2009b).

For the generation of HT-29/B6-GR cells stably expressing the hMR, the hMR CDS was excised from pcDNA3.1/Zeo(+) by PmeI digestion and subcloned into PmeI-cleaved pcDNA3.1/Hygro(+). HT-29/B6-GR cells were stably transfected with pcDNA3.1/Hygro(+)-hMR using Lipofectamine plus (Invitrogen). Screening for clones stably expressing functional hMR was performed by gene reporter assays. The selected clone used for this study is referred to as HT-29/B6-GR/MR.

### *Transfection and gene reporter assay*

For luciferase gene reporter assays, $5 \times 10^5$ cells/6-well were seeded under hormone-free conditions. After 24 h, cells were transfected using Lipofectamine plus (Invitrogen, Karlsruhe, Germany). For the detection of HRE-driven luciferase expression, pMMTV-Luc containing tandem HRE sequences followed by a firefly luciferase (luc) gene served as a reporter gene vector. To study promoter activity of β-ENaC, pbhENaC-A (−1137 to +200) containing the human β-ENaC promoter was used (Amasheh et al., 2004). In the case of the γ-ENaC promoter, the plasmid pghENaC-A (−2926 to +35) was used which was a gift of Dr. Christie P. Thomas (University of Iowa, Iowa City, USA).

As a coreporter construct pGL4.70 [hRluc] (Promega, Mannheim, Germany) containing a *Renilla* luciferase gene was applied. 1 μg of the reporter constructs and 50 ng of the coreporter were used per transfection. 4 h after transfection, aldosterone was added to the cells, in the case of inhibitor experiments preceded by addition of inhibitor substances 1 h earlier. After 24 h, both firefly and *Renilla* luciferase expression was monitored with the Dual-Luciferase Reporter Assay System (Promega) and a Centro LB 960 microplate-luminometer (Berthold, Bad Wildbad, Germany). The *Renilla* luciferase expression level remained unaffected under the experimental conditions and was used to normalize transfection efficiency.

### *Western blot, immunoprecipitation and immunofluorescence*

For the detection of MR and GR, whole cell lysates were prepared by scraping filter-grown cells in whole cell lysis buffer (20 mM Hepes, pH 7.6, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 0.5 μg/ml pepstatin A and 1× Complete protease inhibitor cocktail (Roche, Mannheim, Germany)). Samples were transferred to liquid nitrogen for 5 min, re-thawed and pushed through a 1-ml hypodermic syringe with a 0.45 × 10-mm needle. Whole cell extract was obtained by a final centrifugation (15 min at 14,000 rpm, 4 °C). Protein samples (40 μg for MR and GR) were separated by SDS–PAGE (8.5%) and blotted onto a PVDF membrane. After blocking (5% BSA/PBS-T), membranes were incubated with primary antibodies overnight. Anti-MR antibody MR 1-18 6G1 (1:100) was a kind gift of Dr. Celso E. Gomez-Sanchez (Gomez-Sanchez et al., 2006). Further antibodies were: anti-GR antibody (1:200, Santa Cruz, Heidelberg, Germany) and anti-β-actin (1:10,000, Sigma–Aldrich). For protein detection a Lumi-Light-$^{Plus}$ Western blotting kit (Roche) containing POD-conjugated secondary antibodies and a chemiluminescence system was applied. Chemiluminescence signals were detected using an LAS-1000 imaging system (Fujifilm, Tokyo, Japan).

For the detection of β-ENaC by immunoprecipitation, cells were lysed in the following buffer: 10 mM Tris–Cl pH 7.5, 150 mM NaCl, 0.5% Triton-X-100, 0.1% SDS, 1× Complete protease inhibitor cocktail, 0.2 mM EDTA and 0.2 mM PMSF. Lysis was enhanced by pushing cells through a 1-ml hypodermic syringe with a 0.45 × 10 mm needle. Samples were left on ice for 30 min and lysates were obtained by a final centrifugation (15 min at 15,000 × *g*, 4 °C). 30 or 10 μg of lysate were kept for the detection of β-actin in the input sample in order to assure equal protein load. For immunoprecipitation, 300 μg or 400 μg of lysate protein were incubated with 2 μg of anti-β-ENaC antibody (Alpha Diagnostic, San Antonio, USA) or POD-IgG (goat-anti-mouse-IgG) (Jackson Immuno Research, West Grove, USA) rotating over night in a total volume of 300 μl. 30 μl of 50% protein-A–agarose/PBS slurry were added to each sample which were left rotating at 4 °C for 1 h. After three washing steps with lysis buffer, 30 μl of 2× Laemmli buffer was added to the beads and samples were denatured at 65 °C for 10 min. Protein samples were separated by SDS–PAGE (10%) and Western blot was performed as described above using β-ENaC antibody in a dilution of 1:400.

### *Immunofluorescence*

HT-29/B6-GR-MR cells on permeable supports were fixed with 4% paraformaldehyde for 20 min. Crosslinking was stopped with