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Na^+ absorption defends from paracellular back-leakage by claudin-8 upregulation

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ABSTRACT

In distal colon, the limiting factor for Na^+ absorption is represented by the epithelial sodium channel (ENaC). During absorption, high transepithelial Na^+ gradients are observed. In human colon and in HT-29/B6-GR cells, we investigated whether Na^+ back-leakage is prevented by paracellular sealing.

Tissues and cells were incubated with corticosteroids. Barrier properties were analyzed in electrophysiological experiments. Subsequently, analysis of ENaC and tight junction protein expression, localization, and regulation was performed.

In colon, nanomolar aldosterone induced sodium absorption via ENaC. Concomitantly, paracellular $^{22}Na^+$ permeability was reduced by half and claudin-8 within the tight junction complex was nearly doubled. Real-time PCR validated an increase of claudin-8 transcripts. Two-path impedance spectroscopy following ENaC induction in HT-29/B6-GR revealed a specific increase of paracellular resistance.

These results represent an important physiological implication: Na^+ absorption is paralleled by claudin-8-mediated sealing of the paracellular barrier to prevent Na^+ back-leakage, supporting steep Na^+ gradients in distal colon.

Organs composed of tubular epithelia like intestine and kidney tubules follow a refined strategy for absorbing solutes and water. Compared to proximal parts, the distal segments are characterized by regulated transport, and much tighter tight junctions (TJs) and lower absorption rates which can take place against large electrochemical gradients.

In rat and in human distal colon, Na^+ absorption can be induced by nanomolar aldosterone concentrations added in vitro [1]. This regulation takes place within 8 h on transcriptional level by inducing β- and γ-subunits of ENaC [2,3]. Within this time interval, aldosterone can lower the steady state luminal Na^+ concentration to 2.2 mmol/l, resulting in sodium gradient as low as 1:66 [4]. We presume that this large concentration gradient can only develop if the paracellular pathway is perfectly tightened up, in order to prevent Na^+ to leak back.

Epithelial barrier properties are mainly determined by TJs. Three groups of tetraspan transmembrane proteins located in TJ strands have been described, occludin [5], tricellulin [6], and the family of claudins [7]. Claudins were characterized by a highly variant expression pattern in different epithelia and endothelia [8]. Whereas a lack of occludin did not seem to impair barrier properties [9], the majority of claudins form barriers like claudin-1 [10], claudin-4 [11], claudin-5 [12], and claudin-8 [13]. In contrast, claudin-2 forms ion-specific channels [14]. A segment-specific expression pattern of claudins has been reported in intestine, according to the concept of increasing presence of "tightening" TJ proteins from proximal to distal segments [15].

Having all this in mind, we wondered if aldosterone would not only affect active transcellular transport but could at the same time also influence the paracellular barrier. Claudin-8 has been specifically localized in the TJ of aldosterone-sensitive epithelia [15,16]. Therefore, we included detection of this claudin from the beginning, when we performed respective experiments in human distal colon and in the glucocorticoid receptor-equipped colonic cell line HT-29/B6-GR [17].

Methods

Preparations of human distal colon were obtained from patients undergoing resection of colorectal cancer, as described previously [2]. Electrophysiological experiments were performed as described

Abbreviations: ENaC, epithelial sodium channel; TJ, tight junction.

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before [1–3]. Tissues were mounted in Ussing type chambers, and 3×10^{-9} M aldosterone was added. This concentration is in accordance with plasma levels found in vivo under stimulated conditions [18]. After 8 h of stimulation, addition of amiloride (10^{-4} M) to the apical side caused a drop of short circuit current representing electrogenic Na^+ absorption (J_{Na}; μmol h^{-1} cm^{-2}). In a subset of experiments unidirectional serosal-to-mucosal (s–m) $^{22}Na^+$ fluxes were determined and served as a measure of paracellular Na^+ permeability, because there exists no transcellular secretory pathway for Na^+ in the intestine. For this, after the amiloride-induced drop of short circuit current tissues were short-circuited and $^{22}Na^+$ (23 kBq) was added to the serosal bathing solution. Four 15-min flux periods were analyzed [19], and net flux was calculated as reported before [14]. More details are available in Supplementary file 1.

Cell culture experiments. HT-29/B6 cells, stably transfected with the glucocorticoid receptor (GR-α) were grown to confluence on permeable supports (effective area 0.6 cm^2, Millicell™-HA, Millipore, Bedford, MA) and used on day 7 after seeding as reported recently [17]. After incubation with 1 μM dexamethasone for 48 h, cells were employed for functional and molecular studies.

Two-path impedance spectroscopy. Paracellular (R^{para}), transcellular (R^{trans}), and epithelial resistance (R^e) of HT-29/B6 monolayers were measured by two-path impedance spectroscopy similar to the method described by Reiter et al. [20]. More details are available in Supplementary file 1.

Immunoblots and immunofluorescence microscopy. Immunoblots and Immunofluorescence microscopy were performed as described in detail previously [3,21]. For detection, antibodies raised against TJ proteins (Zymed Inc., San Francisco, CA, USA), and β- and γ-ENaC (Alpha Diagnostic International, San Antonio, TX, USA) were used. More details are available in Supplementary file 1.

Quantitative PCR. Quantitative claudin-8 mRNA determination was performed by real-time PCR employing pre-designed TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). Copy numbers were normalized to GAPDH copies. TaqMan primers were FAM- (Claudin-8) or VIC-labeled (GAPDH). No template controls (NTC), were employed as negative controls. Real-time quantitative reverse transcription-PCR was performed with a 7500/7900HT Fast Real-Time PCR System in conjunction with respective software (SDS2.2.2; Applied Biosystems, Foster City, CA, USA).

Chemicals. If not stated otherwise, all substances were purchased from Sigma (St. Louis, USA).

Statistical analysis. Data are expressed as means ± standard error of the mean with indication of *n* as the number of tissue preparations. Statistical analysis was performed using Student's *t*-test and, if appropriate, Bonferroni–Holm correction for multiple testing. $P < 0.05$ was considered significant.

Results

Induction of electrogenic sodium transport in vitro

Human colon preparations were mounted in Ussing chambers and stimulated with 3 nM aldosterone in vitro. Time course of the measurements revealed a maximum of short circuit current after 8 h ($n = 16$, Fig. 1A). At this time point, 10^{-4} M of the ENaC blocker amiloride was added to the mucosal side. The resulting drop of short circuit current is defined as electrogenic sodium

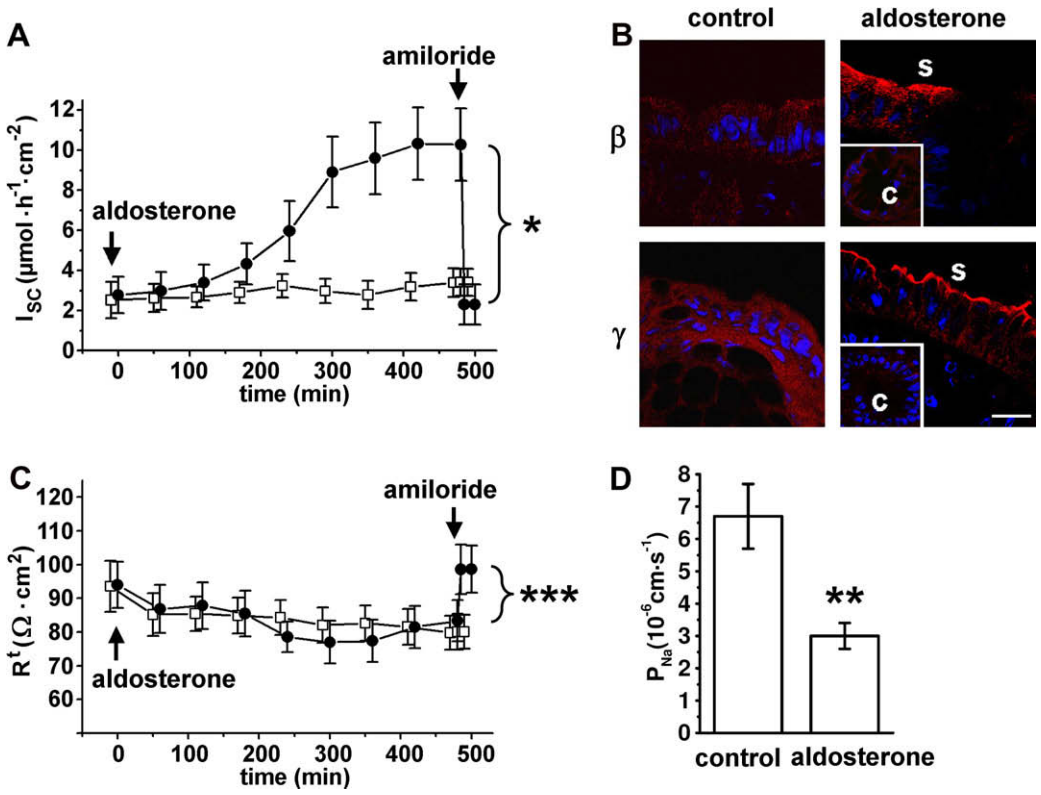

Fig. 1. Induction of the epithelial sodium channel: functional and molecular detection. (A) Time course of I_{SC} after addition of aldosterone ($t = 0$). Tissues were incubated with 3 nM aldosterone in vitro (●) or left unstimulated (□). After 8 h, amiloride (10^{-4} M) was added to determine ENaC-mediated Na^+ absorption. Bracket: resulting aldosterone-induced electrogenic sodium transport J_{Na} ($n = 16$, $^{*}p < 0.05$). (B) Immunofluorescent detection of β- and γ-ENAC induction in the apical membrane of mucosal surface epithelium (s) with no detectable signals in crypts (c; bar: 20 μm). (C) Time course of R^t. Incubation with aldosterone (●) and subsequent inhibition of ENaC by 10^{-4} M amiloride resulted in higher transepithelial resistance R^t compared to unstimulated controls (□). The bracket indicates ENaC contribution to R^t ($n = 16$, $^{***}p < 0.001$). (D) Na^+ permeability (P_{Na}) derived from serosal-to-mucosal $^{22}Na^+$ flux measurements in controls and aldosterone-incubated tissues, measured at the period of final blockade with amiloride ($n = 9$ and 10, $^{**}p < 0.01$).

absorption (J_{Na}), a measure of functional ENaC activation. Whereas controls did not exhibit significant induction of J_{Na} (0.01 ± 0.07 μmol h^{-1} cm^{-2}, in tissues incubated with aldosterone, J_{Na} was 8.0 ± 1.6 μmol h^{-1} cm^{-2} (Fig. 1A, bracket). Subsequently, tissues were removed from the chambers and immunostaining with β- and γ-ENaC antibodies was performed. Confocal laser-scanning microscopy revealed an induction of both subunits in the apical membrane of epithelial cells. Expression was limited to surface cells and no signal was detected in crypts. In controls, no induction was detectable (Fig. 1B).

Transepithelial resistance (R^t) and $^{22}Na^+$ permeability

Time course of R^t is shown in Fig. 1C. Final blockade of ENaC with amiloride caused a rapid change of R^t from 83 ± 6 to 99 ± 7 Ω cm^2. Paired comparison of tissues revealed an increase of 15 ± 2 Ω cm^2 (n = 16, p < 0.001), reflecting the contribution of ENaC (Fig. 1C, bracket). Unstimulated controls revealed no change (−0.03 ± 0.67 Ω cm^2, n = 16). In order to specify this effect, serosal-to-mucosal fluxes of $^{22}Na^+$ were measured at the period of final blockade with amiloride. Because in the colon there exists no transcellular pathway for Na^+ in secretory direction, this flux is proportional to paracellular Na^+ permeability [22]. It turned out that the paracellular permeability for Na^+ in tissues stimulated with aldosterone was reduced by half (P_{Na} 6.7 ± 1.0·10^{-6} cm/s vs. 3.0 ± 0.4·10^{-6} cm/s, n = 9 and 10, respectively, p < 0.01; Fig. 1D).

Detection of TJ proteins in human distal colon

Western blot analyses revealed presence of occludin, claudin-1, -3, -4, -5, -7, and -8 in both, control and aldosterone-incubated tissues (Fig. 2A). The bands corresponded to the expected sizes of 60–64 kD (occludin) and ~22 kD (claudins). After incubation with aldosterone, a marked increase of occludin and claudin-8 signals was observed. Quantification by means of densitometry revealed an increase of occludin to 236 ± 29% and an increase of claudin-8 signals to 179 ± 25% of controls (Fig. 2B, n = 5, p < 0.05). In contrast, claudin-1, -3, -4, -5 and -7 were not significantly different from controls (113 ± 12%, 98 ± 14%, 93 ± 9%, 100 ± 15%, and 91 ± 12%, respectively, n = 4–7, n.s.); claudin-2 was not detectable in the preparations.

Regulation of claudin-8

In order to identify an increase in claudin-8 gene expression as the source of the increased signals of claudin-8, real-time PCR was performed with specific primers and normalized with GAPDH signals. Quantification of nucleic acids revealed a 3.4 ± 0.9-fold increase of claudin-8 mRNA copy numbers after aldosterone incubation, demonstrating induction on transcriptional level of claudin-8 gene regulation (n = 8, p < 0.05, Fig. 2C).

Localization of proteins within the TJ

Immunostainings revealed signals of occludin, claudin-1, -3, -4, -5, -7, and -8 in the apicolateral TJ strand region of epithelial cells. In accordance with Western blot results, occludin signals were more intense in aldosterone-incubated tissues, whereas claudin-1, -3, -4, -5, and -7 remained unchanged. Tissues treated with aldosterone showed no increase of occludin signals within TJs, though. Instead, the increase of occludin fluorescence in aldosterone-treated tissues was limited to basal, thus non-junctional, areas within epithelial cells (Fig. 2D, green). In contrast, claudin-8 was detected

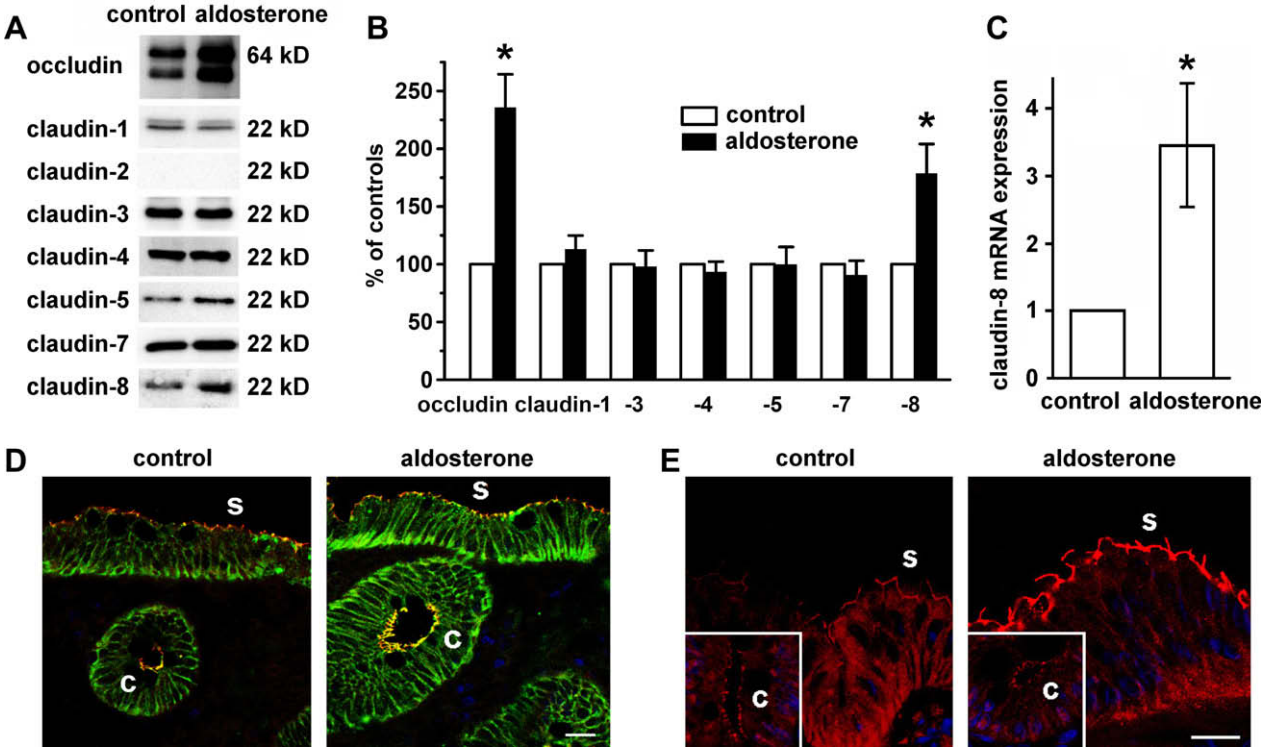

Fig. 2. TJ protein detection in human sigmoid colon during ENaC induction. (A) Western blots. Occludin, claudin-1, -3, -4, -5, -7, and -8 were detected in Western blots in both, controls and aldosterone-incubated tissues, whereas claudin-2 revealed no signals. (B) Densitometry. Signals of occludin and claudin-8 were increased after stimulation with aldosterone, whereas claudin-1, -3, -4, -5, and -7 remained constant (n = 4–7, $^*p$ < 0.05). (C) Real-time PCR of claudin-8. GAPDH-normalized expression of claudin-8 mRNA relative to unstimulated controls. A 3.4 ± 0.9-fold increase of claudin-8 mRNA in colon preparations was detected (n = 8, $^*p$ < 0.05). (D) Confocal laser-scanning microscopy. Occludin (green) and claudin-4 (red). An increase of occludin expression in subjunctional regions was observed in surface epithelia (s) after 8 h stimulation with 3 nM aldosterone (bar: 50 μm). (E) Claudin-8. An increase of claudin-8 signals within the apicolateral membrane of epithelia cells was observed in surface epithelia (s), whereas no increase of signals in crypts (c) was detectable (bar: 20 μm). (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

at higher intensity in TJs of aldosterone-treated tissues compared to controls (Fig. 2E). This increase was strictly limited to surface cells (s) and not present in crypts (c), and therefore exhibited the same distribution between surface and crypts as the aldosterone-induced increase of β- and γ-ENaC (cf. Fig. 1B).

Human colon cell line HT-29/B6-GR

HT-29/B6 cells stably transfected with glucocorticoid receptor (GR) cDNA were analyzed to validate the mechanism. In these cells functional ENaC was induced by 1 μM dexamethasone over a time period of 48 h. In accordance with the results from human colon preparations, Western blots revealed an increase of occludin and claudin-8 after ENaC induction, whereas claudin-4 remained unchanged (126 ± 6%, 132 ± 6%, and 103 ± 7% of controls, $n = 4–8$, Fig. 3A and B). Quantitative PCR revealed a 2.1 ± 0.2-fold increase of claudin-8 mRNA copy numbers ($n = 6$, $p < 0.01$; Fig. 3C). Confocal laser-scanning microscopy revealed an enhanced claudin-8 signal in colocalizion with constant claudin-4 (Fig. 3D). Controls (HT-29/B6) did not show a change of TJ protein expression after incubation with dexamethasone (not shown).

Measurement of paracellular resistance

As a direct proof for the paracellular sealing caused by claudin-8 elevation, two-path impedance spectroscopy was employed (Fig. 3E). Dexamethasone (1 μM) induced in HT-29/B6-GR cells a slight, albeit significant increase in epithelial resistance (R^e; 432 ± 34 vs. 283 ± 15 Ω cm^2, $n = 6$ and 5, respectively, $p < 0.01$) which was lacking when amiloride was present from the beginning of the experiment (311 ± 31 Ω cm^2, $n = 4$). Quantitatively, the effect on R^e was in accordance with R^t obtained in human colon (cf. Fig. 1C). However, as a basis for the observed R^e change, impedance spectroscopy uncovered a strong increase of paracellular resistance after dexamethasone by a factor of nearly three (R^{para}; 2694 ± 34 vs. 935 ± 119 Ω cm^2, $n = 6$ and 5, respectively, $p < 0.01$), which again was prevented in the permanent presence of amiloride (1107 ± 149 Ω cm^2, $n = 4$, n.s.). The transcellular resistance (R^{trans}) did not significantly change, probably due to the relatively small effect of 1 μM dexamethasone on ENaC in this tissue (0.8 μA h^{-1} cm^{-2}, [17]).

Possible signal transduction pathways leading to upregulation of claudin-8

In order to distinguish between a direct aldosterone-mediated effect and Na^+ uptake being the trigger for increased claudin-8 expression, amiloride (10^{-4} M) was added to the tissue prior to addition of aldosterone. In contrast to the protocol in which amiloride was added only at the end of the experiment (cf. Figs. 1 and 2), there was no significant difference in transepithelial resistance and no change of TJ proteins detectable, whereas ENaC induction was not perturbed (Suppl. Fig.).

Discussion

Induction of the epithelial sodium channel proceeds by expression and oligomerization of ENaC subunits in the apical membrane

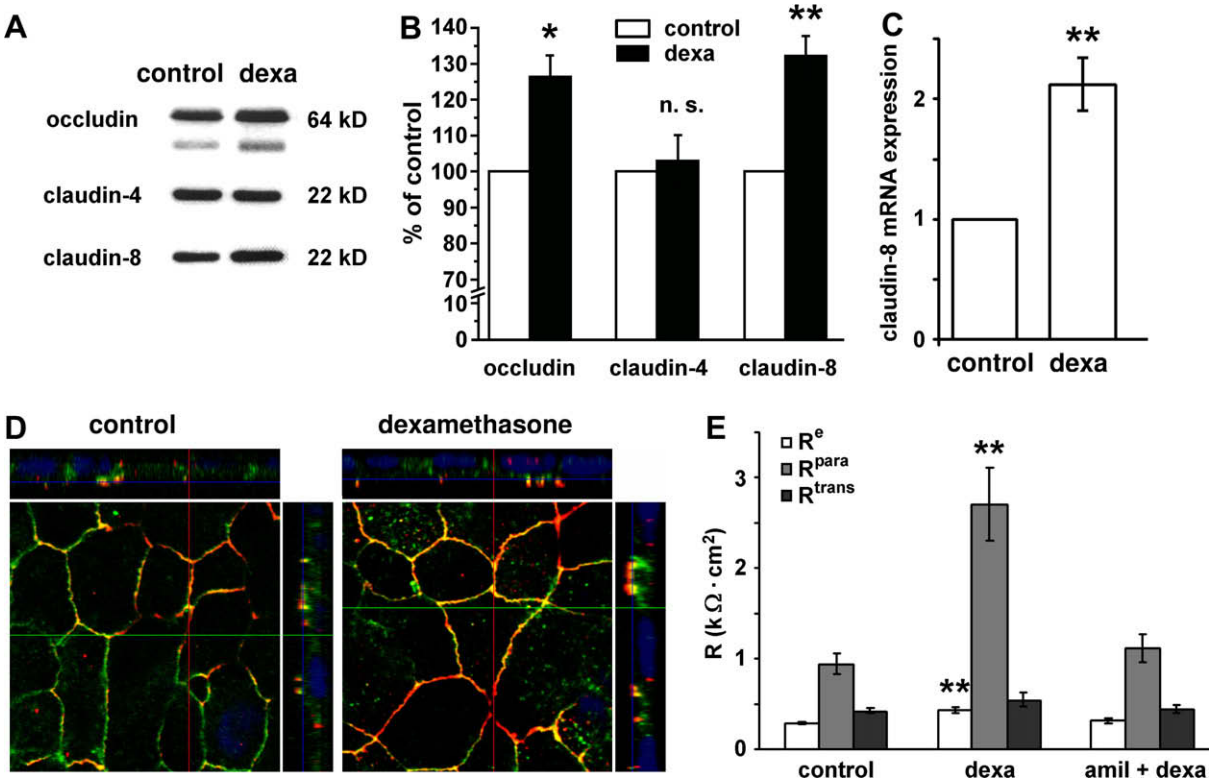

Fig. 3. Effects of ENaC induction on TJ proteins of the colon cell line HT-29/B6-GR. (A) Western blots. (B) Densitometry. Induction of ENaC by dexamethasone (1 μM) resulted in increased signals of occludin and claudin-8, whereas claudin-4 remained constant ($n = 4–8$, $^*p < 0.05$, $^{**}p < 0.01$). TJ protein expression of wild-type cells did not change after incubation with dexamethasone (not shown). (C) Real-time PCR. GAPDH-normalized expression of claudin-8 mRNA relative to unstimulated controls. A 2.1 ± 0.2-fold increase of claudin-8 mRNA in HT-29/B6-GR cells was detected ($n = 6$, $^{**}p < 0.01$). (D) Detection of claudin-4 and -8 in HT-29/B6-GR cells. After incubation with 1 μM dexamethasone, claudin-8 signals (red) were increased within the TJ complex, whereas claudin-4 (green) remained constant. (E) Two-path impedance spectroscopy. Dexamethasone (dexa) induced an increase in epithelial resistance (R^e). This was caused by a strong increase of paracellular resistance (R^{para}), reflecting sealing of the TJ. The dexa-induced increase in R^{para} was prevented if amiloride was present continuously (amil + dexa; $n = 4–6$, $^{**}p < 0.01$). (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

of epithelial cells [2]. The channel consists of the subunits α, β, and γ in a constant stoichiometry [23]. In distal colon, functional ENaC has been demonstrated to be located in the surface epithelium [24]. Molecular ENaC proceeds by transcription of β-ENaC and γ-ENaC, whereas the α-subunit remains constant [2,3]. In our experiments, ENaC induction served as a prerequisite for analyses of TJ protein regulation effected by sodium transport.

Claudin-8 upregulation

Claudin-8 is localized in epithelial TJs of aldosterone-responsive segments of the kidney, namely the distal tubule and collecting duct [16], as well as the colon [25]. Claudin-8 has been demonstrated to increase the cation-selective barrier of epithelia [14,26]. This effect can be achieved either by direct sealing properties [26] or by displacement of pore-forming claudins such as claudin-2 [13]. As claudin-2 was not detected in human distal colon (Fig. 2A; [25]), the latter possibility can be excluded from the beginning.

Occludin and claudins 1–5, and 7

Although the expression of occludin has increased after ENaC induction, this does not necessarily mean that it contributes to the observed increase of transepithelial resistance, for two reasons. First, occludin was not increased within the TJ where it may contribute to barrier function, but only subjunctionally. Second, it was shown previously that occludin knockout does not alter barrier properties in various epithelia [9]. Instead, it had an effect on gastric glandular differentiation pointing to a regulatory role of this protein. Although claudins-1–5, as well as 7 specifically contribute to barrier properties, no changes of these proteins were detected.

Aldosterone and the paracellular barrier

In the very distal segments of tubular epithelia like intestine, nephron, and ducts of excretory glands, aldosterone controls ion transport in order to keep the composition of the body fluids constant. So far, it is consensus that aldosterone does this by altering components of transcellular transport, while the paracellular barrier which prevents back-leakage of the transported solutes is assumed to possess a constant tightness (for review see [27]). However, specific expression in the TJ of aldosterone-responsive segments of kidney and colon already hints at the possibility that claudin-8 regulation may be involved in effective Na^+ net absorption.

Corticosteroid effects on TJ proteins in a variety of cell models have been described recently [28–30]. In our study, direct effects were ruled out by control experiments showing that TJ protein expression was not changed if Na^+ entry is blocked by amiloride from the beginning of ENaC induction. Moreover, experiments were performed with human colon where aldosterone was used which at nanomolar concentration is devoid of glucocorticoid effects, and HT-29/B6 cells using dexamethasone, which demonstrated the same mechanism.

The TJ effect is mediated by enhanced ENaC action

It was beyond the scope of the present study to uncover the full cascade of signal transduction steps between aldosterone or dexamethasone entering the target cell and the increased assembly of claudin-8 into the TJ. Experiments screening for potential signaling pathways were unsuggestive of involvement of common mediators, namely protein kinases PKA, PKC, PKG, MLCK, PI3K, and tyrosine kinases (not shown).

However, we were able to identify the functionally most important step of this cascade in experiments where apical Na^+ entry was permanently blocked by amiloride and no change in TJ protein levels occurred. This means that aldosterone is able to enhance claudin-8 expression only if the ENaC is not only present in the apical membrane but also active. In addition, the present study might provide a key for the understanding of malabsorptive processes which have not directly been linked to single point mutations of transport proteins.

New concept of aldosterone action

Claudin-8 forms also a barrier for divalent cations and protons [31]. Thus we assume that also back-leakage of K^+, H^+, Ca^{2+}, and Mg^{2+} may be reduced. However, the most significant effect concerns Na^+, especially if one considers the large transepithelial gradients of that ion e.g., in large intestine. Rat distal colon develops in vivo (i.e., facing a plasma level of 141 mM) a Na^+ concentration as low as 2.2 mM, yielding a zero-flux lumen-to-plasma ratio of 1:66 [4]. Generation of such an impressive electrochemical gradient would be supported by an increase in tightness of the barrier in order to prevent luminal Na^+ back-leakage. Now we demonstrate that aldosterone indeed stimulates Na^+ transport not only by increased transcellular absorption, but in parallel also by tightening the paracellular pathway against immediate back-leakage of freshly absorbed Na^+.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.10.164.

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