Early aldosterone effect in distal colon by transcriptional regulation of ENaC subunits

H. J. Epple, S. Amasheh, J. Mankertz, M. Goltz, D. Schulzke, and M. Fromm. Early aldosterone effect in distal colon by transcriptional regulation of ENaC subunits. Am J Physiol Gastrointest Liver Physiol 278: G718–G724, 2000.—Aldosterone-induced sodium absorption is mediated by the epithelial Na$^+$ channel (ENaC). It is thought that the "early effect" is not based on genomic regulation of ENaC expression, because ENaC subunit transcription was reported to start later than Na$^+$ transport. We investigated electrogenic Na$^+$ absorption (J$_{\text{ENaC}}$) and, in identical tissues, mRNA expression of ENaC subunits in early (EDC) and late (LDC) distal colon of the rat. In both segments, 8 h in vitro incubation with 3 nM aldosterone enhanced expression of $\beta$- and $\gamma$-ENaC mRNA and induced J$_{\text{Na+}}$. J$_{\text{Na+}}$ was 10 times higher in LDC than in EDC. $\alpha$-ENaC mRNA was unchanged in EDC, whereas it decreased in LDC. In LDC, $\beta$- and $\gamma$-ENaC mRNA was induced 1 h after aldosterone addition, whereas J$_{\text{Na+}}$ became apparent >1 h later. Downregulation of $\alpha$-ENaC mRNA did not take part in acute regulation because it started after a lag time of 3 h. Time correlation of $\beta$- and $\gamma$-ENaC induction and J$_{\text{Na+}}$ stimulation suggests that the early aldosterone effect on Na$^+$ absorption in distal colon is caused by transcriptional upregulation of $\beta$- and $\gamma$-ENaC expression.

Aldosterone is the key hormone in the regulation of Na$^+$ homeostasis. Its Na$^+$-saving action is mediated by the activity of the amiloride-sensitive epithelial Na$^+$ channel (ENaC) located in the apical membrane of aldosterone-responsive tissues such as kidney collecting duct and late distal colon (LDC) (i.e., in human sigmoid and rectum). The importance of proper regulation of ENaC activity is highlighted by human diseases caused by gain or loss of ENaC function due to gene mutations (Liddle disease or pseudohypoaldosteronism type I, respectively) (20). Therefore, the regulation of ENaC activity by aldosterone has been extensively studied by different electrophysiological and biochemical means in a wide variety of mammalian and amphibian epithelia (18).

According to a widely accepted model, ENaC activity [i.e., electrogenic Na$^+$ absorption (J$_{\text{Na+}}$)] in mammalian distal colon is controlled by the mineralocorticoid receptor (MR) (2, 18). Therefore, many functional studies of the action of mineralocorticoid hormones have been performed in this tissue (see, e.g., Refs. 12 and 15). It was demonstrated that the action of aldosterone on Na$^+$ transport is dependent on both intact transcription and translation machinery (for review, see Ref. 17). However, the aldosterone-induced protein(s) responsible for the acute induction of electrogenic sodium absorption have not yet been identified so far (26).

The recent molecular cloning of the three subunits forming the pore of the ENaC (termed $\alpha$, $\beta$, and $\gamma$-ENaC; Refs. 4 and 5) brought new vigor into the search for the molecular mechanism of aldosterone action. Two independent studies found that endogenous aldosterone stimulation enhances $\beta$- and $\gamma$-ENaC mRNA expression in rat distal colon (21, 24). In light of these results, it was suggested that ENaC activity is regulated by aldosterone-dependent transcriptional control of its $\beta$- and $\gamma$-subunits. This straightforward model was challenged by a study on the time course of ENaC mRNA expression in rat distal colon (1). In this study, $\beta$- and $\gamma$-ENaC mRNA was upregulated no earlier than 3 h after the beginning of aldosterone administration. By comparison of data taken from the literature on the time course of J$_{\text{Na+}}$ induced by aldosterone (3, 14–16), it was concluded that the induction of the early response to aldosterone must be independent from $\beta$- and $\gamma$-ENaC mRNA expression (1). Despite the obvious methodological problem of comparing time course data obtained in different laboratories (including our own) under different experimental conditions, the conclusions drawn were generally accepted (2, 29).

Other difficulties that might have distorted the interpretation of molecular or functional data obtained in rat distal colon concern the segmental heterogeneity of this tissue. In all studies on ENaC expression in rat distal colon published so far, the segment of the distal colon taken for the experiments was not further specified, on the assumption that the whole distal colon displays uniform properties. It has been demonstrated, however, that the very distal part of the colon is very sensitive to nanomolar concentrations of the mineralocorticoid, whereas a more proximal segment of distal colon exhibits only minor sensitivity. For that reason, rat distal colon was divided into two functionally distinct segments, termed early (EDC) and late (LDC) distal colon (16). In fact, distinct patterns of subunit
expression have been found along the axis of the renal collecting duct (10).

In light of these results, we studied the effect of mineralocorticoid stimulation on ENaC expression in rat distal colon using a well-characterized in vitro model. Thus we were able to properly define the colonic segment under investigation and to add hormones at specified concentrations without possible interference from the complex regulatory mechanisms present in the in vivo situation. Most importantly, we determined channel activity and ENaC expression in identical epithelia, obtaining functional and molecular data from the same tissues after exactly defined intervals.

METHODS

Preparation of epithelia. Untreated male Wistar rats (200–300 g), fed with a standard rat diet (Altromin 1320) and tap water, were anesthetized and killed by inhalation of ether. The colon was removed, rinsed with Ringer solution, and "totally" stripped of serosa and muscle layers as described previously (16). Two segments of the colon termed LDC and EDC were used in this study. Specimens of LDC were obtained from the very last part of the colon, located between the lymph node at the pelvic brim and the anus. To prepare this extraperitoneal segment of the colon, it was necessary to cut open the pelvic bones. EDC were obtained 6–7 cm proximal to the anus.

Steroid application and electrophysiological measurements. Epithelia were mounted into conventional Ussing-type chambers equipped with water-jacketed gas lifts. The exposed area was 0.54 cm², and the circulating fluid was 10 µl.

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Steroid application and electrophysiological measurements. Epithelia were mounted into conventional Ussing-type chambers equipped with water-jacketed gas lifts. The exposed area was 0.54 cm², and the circulating fluid was 10 µl. The bathing solution consisted of (mM) 140.5 Na⁺, 5.4 K⁺, 1.2 Ca²⁺, 1.2 Mg²⁺, 123.8 Cl⁻, 21 HCO₃⁻, 2.4 HPO₄²⁻, 0.6 H₂PO₄, 10 α(+)-glucose, 10 α(+)-mannose, 0.5 β-OH-butyrate, 2.5 glutamine, and 50 mg/l azlocillin (Securopen, Bayer).

Short-circuit current (I_{sc}, µmol·h⁻¹·cm⁻²) and tissue conductance (mS/cm²) were recorded using microprocessor-driven clamp devices (AC-microclamp, f. p. Datensysteme, Aachen, Germany). The resistance of the bathing fluid between the voltage-sensing electrodes was measured before each experiment and taken into account. After mounting of the tissues and a 30-min equilibration period, steroids and other substances were added to both sides of the epithelium as indicated. For time course experiments, all epithelia including controls were kept in the Ussing chamber for 8 h (after equilibration) before J_{Na} was determined. During this time, aldosterone was added to the bathing solution so that the indicated incubation times were achieved. All substances used were dissolved in 96% methanol so that 10 µl added to the bathing solution gave the desired final concentration. Methanol alone had no detectable effect on I_{sc} and mRNA expression. At the end of the electrophysiological measurement, amiloride (10⁻⁴ M) was added to the mucosal compartment. The drop in I_{sc} after addition of amiloride was assigned to J_{Na}. Thus all results of amiloride-sensitive I_{sc} are expressed as fluxes of monovalent cations (µmol·h⁻¹·cm⁻²).

Extraction and isolation of RNA. RNA was prepared from matched groups of epithelia (untreated controls and epithelia treated as specified) immediately after determination of J_{Na}. The area of the epithelium exposed to the bathing fluid was cut out with a scalpel. For RNA extraction, three identically treated epithelia were pooled and placed into 6 ml of iced RNAzol (Boehringer Mannheim). The epithelia were then homogenized using an Ultra-Turrax (Ika-Werk, J anke &Kunkel). Extraction of total RNA was performed according to the instructions given by the manufacturer. In this way, 60–120 µg of total RNA could be extracted from three tissue preparations.

Northern hybridization. Aliquots of 10 µg of colon epithelia total RNA were separated on 1% agarose gels in 1× MOPS under denaturing conditions (2% formaldehyde). Nuclear acids were transferred to nylon membranes (Boehringer Mannheim) and ultraviolet cross-linked. The membranes were hybridized for 2 h at 68°C in Quik-Hyb (Stratagene) and 100 µg/ml herring sperm DNA with digoxigenin-labeled cDNA probes corresponding to α-, β-, or γ-ENaC and glyceroldehyde-3-phosphate dehydrogenase (GAPDH). After stringency washes (2× SSC-0.1% SDS at room temperature and 0.5× SSC-0.1% SDS at 65°C, 2 times each), probes for ENaC subunit mRNA and GAPDH mRNA were detected using anti-digoxigenin antibody conjugated to alkaline phosphatase (Anti-Digoxigenin-AP, Boehringer Mannheim) and a chemiluminescent substrate (CDP-Star, Boehringer Mannheim) according to the instructions of the manufacturer. Hybridization intensity was quantified with illumincent imaging (LAS-1000, Fujifilm) using AIDA software (Raytest). The intensity of the GAPDH signal was used for normalization to detect differences between different lanes.

PCR. Probes were prepared by PCR with digoxigenin-labeled dUTP using a PCR digoxigenin probe synthesis kit (Boehringer Mannheim). cDNA clones for α-, β-, γ-ENaC were kindly provided by B. Rossier (Dept. of Pharmacology, University of Lausanne, Lausanne, Switzerland), and cDNA clone for rat GAPDH was kindly provided by O. Huber (Inst. of Clinical Chemistry, Freie Universität Berlin, Berlin, Germany). Sense and antisense primers were designed based on regions that showed significant sequence divergence between the respective ENaC subunits. The primer pairs were 5’-CACAGCATGTGACATTAC-3’ (sense) and 5’-AGTGT- CACAGGAGCTGAC-3’ (antisense), extending from bases 1397 to 1416 and 1814 to 1795 of α-ENaC; 5’-CGGCTCCGCAG- GTTGGACATCC-3’ (sense) and 5’-CTCTGTTCCGTCCTG- GAGACAG-3’ (antisense), extending from bases 1158 to 1177 and 1510 to 1498 of β-ENaC; 5’-CTCAAGCAGTGTGACATTAC- TGTTGACATCC-3’ (sense) and 5’-TGGGAATCCATTTCGTGCAG- GTTGG-3’ (antisense), extending from bases 2388 to 2412 and 2695 to 2671 of γ-ENaC; and 5’-GACACTCTCCATTTGTCAC- GATTGACATCC-3’ (sense) and 5’-TTTCCTGTGACATCCATAC- TGTC-3’ (antisense), extending from bases 445 to 467 and 804 to 781 of rat GAPDH.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed using Student’s t-test. P < 0.05 was considered significant.

RESULTS

Effect of aldosterone on rat EDC and LDC. After 8-h in vitro stimulation with 3 nM aldosterone, which is a concentration found in vivo under stimulated conditions (14), J_{Na} was determined by mucosal addition of 10⁻⁴ M amiloride (Fig. 1A). As described previously (16), J_{Na} in LDC was >10 times that of EDC (Fig. 1B; 14.2 ± 2.1 and 1.25 ± 0.4 µmol·h⁻¹·cm⁻², respectively, n = 9 each), confirming the concept of functional
diversity within the distal colon. In controls, there was no significant $J_{\text{Na}}$ in either EDC or LDC.

Directly after completion of the electrophysiological measurements, RNA was extracted from the tissues for northern blot hybridization. In aldosterone-treated epithelia a marked increase of $\beta$- and $\gamma$-ENaC mRNA expression was found in both EDC and LDC (Fig. 2; $P < 0.001$, $n = 9$). In contrast to earlier in vivo studies (1, 21, 24), $\alpha$-ENaC was not constitutively expressed in LDC. Instead, in this segment of the distal colon, $\alpha$-ENaC mRNA was downregulated after 8 h of aldosterone incubation (65.7 $\pm$ 10% of controls, $P < 0.05$, $n = 12$; Fig. 1C). In EDC on the other hand, $\alpha$-ENaC mRNA remained unchanged with or without aldosterone (aldosterone-treated EDC: 112.3 $\pm$ 12.4% of controls, $n = 9$).

To further characterize this surprising finding, we investigated the effect of different aldosterone concentrations on expression of $\alpha$-ENaC mRNA in EDC and LDC. Aldosterone was varied between 0.1 nM and 0.3 $\mu$M, because it has been shown that $J_{\text{Na}}$ is regulated by aldosterone within this concentration range (16). In EDC, the expression of $\alpha$-ENaC mRNA remained unchanged compared with controls. In LDC however, 0.1 nM aldosterone sufficed to maximally suppress $\alpha$-ENaC expression (Fig. 3). The different expression of $\alpha$-ENaC mRNA in EDC and LDC added to the concept of a segmental heterogeneity within the distal colon.

Timing course. To achieve maximal resolution, we performed time course experiments in LDC. Epithelia were incubated with aldosterone for the time period indicated. After determination of $J_{\text{Na}}$, RNA was extracted from the epithelia for quantification of ENaC expression as described in METHODS. Thus the molecular data obtained reflect the RNA expression at the time of the $J_{\text{Na}}$ measurement.

As shown previously (16), $J_{\text{Na}}$ started to increase between 2 and 3 h after addition of aldosterone, reaching maximum values 8 h after aldosterone incubation (Fig. 4). Interestingly, $\beta$- and $\gamma$-ENaC mRNA was found to be increased as soon as 1 h after aldosterone addi-
tion, more than 1 h before $J_{\text{Na}}$ started to rise (Fig. 5). This result is clearly compatible with a model of acute regulation of ENaC activity by transcriptional control of its $\beta$- and $\gamma$-subunit expression.

The downregulation of $\alpha$-ENaC mRNA only started after a lag time of 3 h, when $J_{\text{Na}}$ has already started to increase (Fig. 5). Thus $\alpha$-ENaC apparently does not take part in the acute regulation of ENaC activity in this tissue. In further experiments, therefore, we concentrated on the correlation between $J_{\text{Na}}$ and expression of $\beta$- and $\gamma$-ENaC only.

Heterodimeric activation of ENaC mRNA expression. In a recent study, we demonstrated (19) stimulation of $J_{\text{Na}}$ in rat distal colon by the activated glucocorticoid receptor (GR) and cooperative stimulation of $J_{\text{Na}}$ by heterodimers of activated MR and GR. To investigate the effect of heterodimeric activation on ENaC mRNA expression, we combined a MR-specific concentration of aldosterone (0.1 nM) with a GR-specific concentration of the "pure" glucocorticoid RU-28362 (1 nM) in the same experiment. In these concentrations, aldosterone induced small $J_{\text{Na}}$ (1.3 ± 0.3 µmol·h⁻¹·cm⁻²; $P < 0.001$, $n = 8$), whereas RU-28362 did not induce significant $J_{\text{Na}}$ (0.3 ± 0.2 µmol·h⁻¹·cm⁻²; not significant, $n = 7$). The combination of both hormones evoked a clearly overadditive response ($J_{\text{Na}}$ 5.8 ± 1.0 µmol·h⁻¹·cm⁻², $n = 8$) as reported previously (19) (Fig. 6A).

This positive cooperativity was reflected at the molecular level. Although aldosterone and RU-28362 only induced a small increase of $\beta$- and $\gamma$-ENaC mRNA when
Given alone, the combination of both substances strongly enhanced β- and γ-ENaC mRNA expression (Fig. 6B).

**Discussion**

Time course. Despite substantial progress in the last few years, the molecular mediation of the acute action of aldosterone in mammalian distal colon is still unknown. Because this action is dependent on functional transcription and translation (17), several aldosterone-induced proteins have been proposed as potential mediators inducing $J_{\text{Na}}$ (see, e.g., Refs. 7, 11, 23, 25). However, the mRNA encoding for these proteins has been shown to be induced too late to explain the "early" action of aldosterone. On the other hand, "early" aldosterone-induced RNAs or proteins were identified by differential display PCR in the toad kidney cell line A6, but the regulatory role of these aldosterone-induced proteins in the intact mammalian target organ has not been elucidated so far (6, 26).

After the successful cloning of the cDNA encoding for the subunits composing the ENaC (4, 5), these subunits were obviously key candidates as possible mediators of...
the aldosterone response. Three studies investigated aldosterone-dependent ENaC subunit expression in rat distal colon (1, 21, 24). All of them made use of aldosterone stimulation in vivo. Additional electrophysiological measurements for ENaC activity were only performed in one study, demonstrating a parallel increase of γ-ENaC expression and JNa after 14 days of salt deprivation, whereas α-ENaC mRNA remained unaltered (21). This result seemed to indicate a regulation of ENaC activity by transcriptional control of its γ-subunit (expression of β-ENaC was not investigated in this study). However, a subsequent study using implanted minipumps failed to demonstrate a significant increase in ENaC subunit mRNA expression during the first 3 h of aldosterone administration (1). Although no measurement of ENaC function was performed, the time course obtained in this study was used as an argument against a direct transcriptional control of ENaC activity in the acute action of aldosterone (2). Consequently, it was concluded that the short-term response to aldosterone is mediated not via expression of ENaC subunits but rather by different "early" aldosterone-induced regulatory proteins (6, 26, 29).

The results of the present study disprove these conclusions. The time courses of β- and γ-ENaC mRNA induction and JNa increase clearly demonstrate that the JNa increase lags behind that of β- and γ-ENaC mRNA expression and not vice versa as proposed earlier (1). Thus the time courses of β- and γ-ENaC mRNA expression and JNa found in the present study are entirely compatible with direct transcriptional control of ENaC activity by expression of its β- and γ-subunits, although our results do not, of course, exclude the participation of other mechanisms in the regulation of ENaC function. In this context, however, it should be mentioned that the time course of ENaC RNA induction in vitro presented in the present study is in accordance with former in vivo analysis of Na+ transport using the same colonic segment and a similar aldosterone concentration (13).

The experimental model used in the present study is probably better suited to resolve the time correlation between ENaC activity and mRNA expression during the acute aldosterone response than the models of earlier studies. Because in our study electrophysiological and molecular data were obtained from identical epithelia, these data reflect the functional state of the epithelial at a given time. One explanation for the delayed effect of previous in vivo studies using minipump infusion may be the only gradual increase of aldosterone concentrations at the target cells, which is slow compared with the immediate delivery in our in vitro setup. In the latter case, it is very difficult to estimate the time needed to reach an equilibrium of receptor occupancy. Thus conclusions based on correlation of molecular and functional time course should only be drawn if these data were obtained simultaneously using the same experimental system.

α-Subunit. In EDC α-ENaC was constitutively expressed independent from aldosterone as described for rat distal colon previously (1, 21, 24). In contrast, in LDC we observed aldosterone-dependent downregulation of α-ENaC mRNA. This is the first report of aldosterone-dependent downregulation of an ENaC subunit in the colon. To the best of our knowledge, there is only one study, using a rat parotid cell line, that reports downregulation of α-ENaC in a mammalian tissue (30). In this study a phorbol ester [12-O-tetradecanoylphorbol 13-acetate (TPA)] repressed α-ENaC expression, and this effect was abolished by pretreatment of the cells with an inhibitor of the extracellular signal-regulated protein kinase pathway (PD-98059; Ref. 30). In our experimental model using intact distal colon, however, neither TPA nor PD-98059 had any effect on α-ENaC expression (data not shown), suggesting different regulatory pathways for α-ENaC mRNA expression in rat distal colon and parotid cells. For two reasons, a regulatory role of α-ENaC in the acute response of the distal colon to aldosterone seems unlikely. First, downregulation of α-ENaC clearly occurred only after JNa was already induced. Second, α-ENaC mRNA was maximally suppressed by 0.1 nM aldosterone, which was shown to exert no measurable effect on JNa in a previous study (16).

Segmental heterogeneity. The functionally defined segmental heterogeneity of the distal colon with an aboral gradient of increasing aldosterone sensitivity (16) was extended by the different patterns of α-ENaC mRNA expression found in EDC and LDC. In the present study α-ENaC mRNA downregulation was found only in the most distal part of the LDC located between the anus and the lymph node regularly present at the pelvic brim [a segment termed LDC2 in a previous study from our laboratory (16)]. The reason why earlier in vivo studies failed to observe downregulation of α-ENaC may be that RNA was extracted from more proximal parts of the colon.

Heterodimeric activation. According to a generally accepted model, JNa in the distal colon can only be induced by activated MR (2, 18). However, two recent studies demonstrated stimulation of JNa by the GR-specific substance RU-28362 (19, 22). Moreover, it was shown that the combination of RU-28362 with aldosterone exerts a synergistic action on JNa, if receptor-specific concentrations of the respective hormones are used. To explain these results, heterodimerization between MR and GR was proposed (19). In the present study, we found positive cooperativity also on the molecular level. Receptor-specific concentrations of aldosterone and RU-28362 revealed a clearly cooperative effect on both JNa and β- and γ-ENaC mRNA expression. This finding indicates that the cooperative process takes place between receptor binding of the hormones and transcription of the β- and γ-ENaC genes. It is well described that activated steroid receptors bind as dimers to the steroid-responsive elements (27, 28) and that the process of dimerization and receptor binding is highly cooperative (8). Therefore, it seems very likely that the positive cooperativity of aldosterone and RU-28362 on JNa and ENaC subunit mRNA expression is caused by MR and GR heterodimerization.
As to the quantitative effects of aldosterone and the combination of aldosterone with RU-28362, the magnitude of \( J_{Na} \) was fully accounted for by different levels of \( \beta \)- and \( \gamma \)-ENaC mRNA expression. In fact, under all experimental conditions used in the present study, independent of the respective hormones and combinations used or the colonic segment investigated, \( \beta \)- and \( \gamma \)-ENaC mRNA expression always paralleled and preceded \( J_{Na} \). Thus, although other mechanisms may also be involved, ENaC expression must be considered as a key candidate mechanism in the acute regulation of ENaC function in the distal colon.

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