ALDOSTERONE REGULATED SODIUM TRANSPORT in the KIDNEY

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Aldosterone regulated sodium transport in the kidney

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

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CHAPTER 1

General Introduction
Chapter 1

General Introduction

The main factors influencing blood pressure are cardiac output, peripheral resistance, and Na⁺ balance. The short-term regulation of blood pressure, mediated by the nervous system and hormones in the circulation, counteract moment to moment fluctuations in blood pressure by altering peripheral resistance and cardiac output. By contrast, the long-term regulation of blood pressure is realized via the slower-acting renal mechanisms, regulating the Na⁺ balance. The blood Na⁺ concentration is tightly controlled by the amount of Na⁺ reabsorption in the distal nephron of the kidney. Since reabsorption of Na⁺ is accompanied by water reabsorption, blood volume can be controlled by regulating the Na⁺ balance of our body.

Body fluid composition

Total body water, 50-60% of the body weight, is a function not only of weight, age and sex but also of the relative amount of body fat. Of all tissues, adipose tissue is least hydrated (containing up to 20% water). By contrast, skeletal muscle mass contains about 65% water. Body water exists in two main compartments: 1) the intracellular fluid, which contains two thirds of the total body water; and 2) the extracellular fluid, which contains the remaining one third. Extracellular fluid is located in many separated spaces of the body. The two main subcompartments are interstitial fluid and blood, which consist of approximately 75% and 25% of the extracellular fluid, respectively. The dominant cation of extracellular fluid is Na⁺, and the major anions are Cl⁻ and HCO₃⁻. It is essential to maintain a constant extracellular fluid volume in the face of marked variations in water and salt uptake. In contrast to the extracellular fluid, the [Na⁺] in the intracellular fluid is extremely low. The most abundant cation in the intracellular fluid is K⁺, and its major anion is HPO₄²⁻. The characteristic distribution of the Na⁺ and K⁺ ions on the two sides of cellular membranes reflects activity of ATP-dependent Na⁺, K⁺-pumps, which keep [Na⁺], low while maintaining high [K⁺].
The Kidney

The kidney plays a crucial role in body fluid and electrolyte homeostasis. To perform this duty, the functional unit of the kidney, the nephron, is highly specialized and shows a complex longitudinal differentiation of reabsorbing segments that are made up of the following epithelial compartments: 1) the proximal nephron, with its straight (PST) and convoluted tubule (PCT); 2) intermediate tubule, consisting of the descending thin limb (DTL) and the ascending thin limb (ATL); 3) the distal nephron, which comprises the thick ascending limb (TAL) of Henle’s loop and the distal convoluted tubule (DCT); 4) the collecting system with the connecting tubule (CNT), the cortical, outer medullary, and inner medullary collecting ducts (CCD, OMCD, IMCD) (see Fig. 1) (Kriz, 1988).

Figure 1: Organization of the nephron. A nephron is illustrated; G, glomerulus; PST, proximal straight tubule; PCT, proximal convoluted tubule; DTL, descending thin limb; ATL, ascending thin limb; TAL, thick ascending limb; DCT, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct. (Adapted from Berne and Levy, 1993)
Each kidney contains approximately 1.2 million nephrons. Per day, about 180 liter of fluid is filtered in the glomeruli, the initial filtration units of the nephron. This ultrafiltrate or primary urine is cell free and almost protein free, but it contains all the plasma solutes in virtually the same concentration as in plasma. Reabsorption of the most useful solutes and water is near 100%, so that the amounts excreted in the urine represent a very small fraction (less than 1%) of the ultrafiltrate. The capacity to regulate the excretion of water and solutes, enables the kidney to control the extracellular circulating volume and its osmolarity, within a very narrow range despite large variations in the intake of water and solutes.

**Proximal Tubule**

The filtration rate of 180 L/day corresponds to approximately 1.7 kg/day of NaCl. The main fraction of Na⁺ and water reabsorption takes place in the proximal tubules (about 60-70%). The key energy providing element in proximal tubule reabsorption is the Na⁺,K⁺-ATPase, the Na⁺ pump in the basolateral membrane. NaCl and water reabsorption by the proximal tubule can be divided in two phases. During the first phase of proximal tubule reabsorption, Na⁺ uptake is coupled to glucose, amino acids, PO₄, lactate and HCO₃⁻. This entry into the cell, across the apical membrane, is mediated by specific symporter and antiporter proteins, with the Na⁺/H⁺-exchange as the most prominent Na⁺-coupled transporter. In the second phase of proximal tubule reabsorption, Na⁺ is taken up mainly with Cl⁻ as accompanying anion via both the transcellular and paracellular pathways.

**Henle’s Loop and Distal Convoluted Tubule**

Henle’s loop reabsorbs about 20-30% of the filtered load of Na⁺ and Cl⁻. This reabsorption is driven by the basolateral Na⁺,K⁺-ATPase, which provides a chemical gradient for the movement of Na⁺ into the cell by maintaining a low intracellular Na⁺ concentration. Na⁺ is taken up by the apically located furosemide-sensitive Na⁺,2Cl⁻,K⁺ cotransporter. K⁺ recycling via ROMK-type channels together with Cl⁻ exit are used to drive Na⁺ across the paracellular pathway. The distal convoluted tubule reabsorbs approximately 5-7% of the filtered load of Na⁺. The bulk of transcellular absorption of Na⁺ and Cl⁻ is accomplished by the electroneutral thiazide-sensitive Na⁺-Cl⁻ symporter. The Na⁺
reabsorption in Henle’s loop as well as in distal convoluted tubule is largely dependent of the concentration on $\text{Na}^+$ in the luminal fluid.

Connecting Tubule and Collecting Duct

The connecting tubule and cortical collecting duct have a more limited reabsorption capacity. However, in these distal segments the fine tuning in regulating the composition and volume of the urine takes place. The CCD is composed of three different cell types, the principal cells and two types of intercalated cells (designated $\alpha$ and $\beta$): the principal cells are the dominant cell types, the intercalated cells constitute approximately 30% of the cells in these segments. The principal cells reabsorb $\text{Na}^+$ and water and secrete $\text{K}^+$, whereas the intercalated cells reabsorb $\text{HCO}_3^-$ and secrete $\text{H}^+$. In the collecting tubule and collecting duct, $\text{Na}^+$ enters the cell apically via the highly selective epithelial $\text{Na}^+$ channel (ENaC) and the basolateral $\text{Na}^+$ extrusion is entirely dependent on $\text{Na}^+,\text{K}^+$-ATPase (Fig. 2). In this thesis we studied $\text{Na}^+$ reabsorption in the CNT/CCD and, in particular the regulation of ENaC in these cells.

![Figure 2: Transport mechanism for $\text{Na}^+$ reabsorption in the principal cell of the cortical collecting system](image)

*Figure 2: Transport mechanism for $\text{Na}^+$ reabsorption in the principal cell of the cortical collecting system*
Epithelial Na\(^+\) channel (ENaC)

The epithelial Na\(^+\) channel, ENaC mediates the electrogenic influx of Na\(^+\) across the apical CNT and CCD membranes into the cell and down its electrochemical gradient. Besides the distal kidney tubules, this channel has been identified in distal segments of the colon, in airway epithelium, skin, urinary bladder, sweat glands and salivary glands (Lingueglia et al., 1996). When the first subunit of ENaC was identified (Canessa et al., 1993), it was found to be related to a group of genes, called degenerins (DEG), expressed in the nervous system of the nematode \textit{C. elegans}. Based on these first two identified subfamilies, this ion channel class is called the DEG/ENaC superfamily (Mano and Driscoll, 1999). DEG/ENaC channels have been implicated in a broad spectrum of cellular functions, including touch sensation, coordination, pain sensation, gametogenesis and blood pressure regulation. Members of the DEG/ENaC superfamily have been identified in nematodes, snails, flies, and several vertebrates, including mammals.

ENaC is blocked with high affinity by amiloride and the amiloride analogs, like benzamil. Furthermore, ENaC has a low single channel conductance (3-6 pS) and a high selectivity for Na\(^+\) and Li\(^+\) and is impermeable for K\(^+\) (Garty et al., 1994). It exhibits a high open probability and long open channel times (Garty and Palmer, 1997). The heteromultimeric ENaC is formed by three structurally related subunits \(\alpha\), \(\beta\) and \(\gamma\) ENaC which share an amino acid identity of about 34-37\% (Canessa et al., 1994). Like all members in the DEG/ENaC superfamily, each subunit has a large extracellular loop, two short membrane-spanning domains and short cytoplasmic NH\(_2\) and COOH termini (Fig.3) (Renard et al., 1994). The NH\(_2\) and COOH terminal domains of the subunits contain potential regulatory elements, such as consensus sites for phosphorylation by protein kinase C and conserved proline-rich motifs (Canessa, 1996). The conserved proline-rich motifs in the C terminal region of the \(\alpha\) ENaC subunit are essential for apical localization of the channel, whereas the proline-rich motifs in the C terminal region of the \(\beta\) and \(\gamma\) ENaC subunits are essential for stability of the channel (Rotin et al., 1994; Staub et al., 1997). The extracellular domain is glycosylated at 6, 12 and 4-5 asparagine residues in \(\alpha\), \(\beta\) and \(\gamma\) ENaC, respectively (Canessa, 1996). Furthermore, in each subunit a cysteine-rich domain is found in the extracellular domain, but its function is still unknown.
In the *Xenopus laevis* oocyte expression system the α ENaC subunit is sufficient to induce channel activity, whereas coexpression of α ENaC with β and γ ENaC subunits are necessary to obtain maximal channel current (Canessa et al., 1994). However, the number of subunits and stoichiometry of the hetero-oligomeric ENaC complex is still a matter of debate. Biochemical and biophysical studies suggested two different subunit compositions: a tetrameric structure consisting of two α, one β and one γ ENaC subunits (Firsov et al., 1998; Kosari et al., 1998) or an eight to nine hetero-oligomer (Cheng et al., 1998; Eskandari et al., 1999; Snijder et al., 1998), respectively.

*Control of sodium reabsorption*

ENaC plays an important role in Na\(^+\) homeostasis and blood pressure regulation, therefore it is tightly controlled by numerous factors. It is upregulated by hormones such as aldosterone and vasopressin and are under feedback control of intracellular Na\(^+\) concentrations ([Na\(^+\)]\_i). The apical membrane Na\(^+\) permeability is downregulated by inhibiting the basolateral Na\(^+\) extrusion (Turnheim et al., 1991). The function of this feedback loop is to prevent large increases in [Na\(^+\)]\_i and cell volume. Furthermore, in recent years several accessory regulatory proteins have been identified, which are implicated in the
regulation of ENaC activity. The suppressor protein Nedd4 and activator protein sgk will be described in more detail.

**Aldosterone**

The steroid aldosterone plays a major role in the regulation of ENaC function, but the mechanisms involved are not completely understood. Aldosterone secretion is stimulated by a decrease in plasma volume and as a consequence by low blood pressure. Early studies have revealed that aldosterone increased both, the passive apical Na⁺ influx into epithelial cells and active Na⁺ extrusion into the blood (Garty, 1992; Verrey, 1995). Under most conditions, the rate limiting step of Na⁺ transport in the distal nephron is the Na⁺ entry via ENaC.

Three basic mechanisms are possibly involved in aldosterone stimulated Na⁺ transport: first, synthesis and insertion of ENaC into the plasma membrane; second, activation of existing channels already in the plasma membrane; and third, increasing the open probability of ENaC. It is not clear whether the effects of aldosterone result from direct regulation of the channel or whether they are secondary to aldosterone-induced (or suppressed) proteins. The cloning of the three ENaC subunits has enabled the study of aldosterone regulation on ENaC mRNA and protein level. Surprisingly, the three subunits can respond differently to aldosterone. The subunits can also respond differently in the various aldosterone-responsive tissues and differs among different mammals (Linguelia et al., 1994; Renard et al., 1995; Stokes et al., 1998).

In toad bladder and the amphibian A6 cell line the response to aldosterone can be divided in three phases. The first period is a lag phase of 30-90 min. In the second phase, Na⁺ transport increases and the transepithelial electrical resistance drops. Finally, in the third phase from 3-24 h the Na⁺ transport further increases (Garty and Benos, 1988; May et al., 1997). In A6 cells, the early response is accompanied by an increase in α ENaC protein level. The protein level of β ENaC is increased 6 h after aldosterone addition, whereas the γ ENaC protein level remains constant. In contrast to the various aldosterone effects on α, β and γ ENaC protein levels, the ENaC mRNA levels for all the three subunits are only induced after long-term aldosterone stimulation.
Vasopressin

The antidiuretic hormone, vasopressin increases water reabsorption but also an effect on Na⁺ reabsorption has been demonstrated (Blot-Chabaud et al., 1996; Frindt et al., 1972; Granthamm et al., 1966; Gross et al., 1975; Tomita et al., 1985). The vasopressin stimulated effect is observed within 5-20 min as a two to fourfold increase in Na⁺ transport. This short-term effect of vasopressin is transient. In addition to this classical short-term effect, on RCCD (rat cortical collecting duct cell line) an increase in both short-circuit current and ²²Na⁺ transport was observed after 6 h vasopressin exposure (Djelidi et al., 1997). Interestingly, Reif and co-workers (1986) demonstrated in perfused rat cortical collecting tubules that the increase in Na⁺ transport by vasopressin is additive to the aldosterone stimulated effect.

Serum and glucocorticoid-regulated kinase (sgk)

Sgk, a serine-threonine kinase was identified as an early aldosterone-induced protein (Chen et al., 1999). In rat and rabbit cortical collecting tubules and in A6 cells, aldosterone increases the sgk mRNA and protein levels within 30 min after addition of the hormone (Náray-Fejes-Tóth et al., 1999; Chen et al., 1999). Moreover, coexpression of sgk and ENaC in *Xenopus laevis* oocytes showed an increase in amiloride-sensitive Na⁺ transport by approximately four to sevenfold (Shigaev et al., 2000; Chen et al., 1999) compared with ENaC expressed alone. Aldosterone increases the phosphorylation of the serine/threonine residues in the C termini of the β and γ ENaC (Shimkets et al., 1998) in MDCK cells (a cell line derived from canine distal tubule of the kidney). However, Alvarez de la Rosa and co-workers (1999) postulated that the aldosterone-induced phosphorylation of ENaC is not mediated directly through sgk.

Nedd4

Nedd4 (Neuronal precursor cells Expressed Developmentally Downregulated) protein originally cloned from mouse brain (Kumar et al., 1992), was identified as interacting
partner of ENaC (Staub et al, 1996). Nedd4 is a ubiquitin protein ligase, and in addition to the ubiquitin ligase (Hect) domain, Nedd4 also contains three or four WW domains. The interaction between Nedd4 and ENaC occurs by binding of these WW domains with the proline-rich motifs in the C-termini of ENaC subunits. Coexpression of Nedd4 and ENaC in *Xenopus laevis* oocytes inhibited ENaC activity by reducing the number of ENaC channels at the cell surface (Abriel et al., 1999). This downregulation of ENaC by Nedd4 is mediated by ubiquitination of the channel, leading to endocytosis and degradation in the proteasome (Goulet et al., 1998).

**Genetic disorders of ENaC**

The importance of ENaC for the regulation of sodium balance, blood volume and blood pressure is strikingly illustrated by mutations in genes coding for the subunits of ENaC that cause two human syndromes, Liddle’s syndrome and pseudohypoaldosteronism. Mutations causing Liddle’s syndrome result in a gain of function and therefore, lead to hypertension. Whereas mutations causing pseudohypoaldosteronism result in a channel loss of function of ENaC and are associated with hypotension.

**Liddle’s syndrome**

Liddle’s syndrome is an autosomal dominant form of hypertension, characterized by early onset of hypertension associated with excessive K+ urinary loss, resulting in hypokalemia, (Liddle et al., 1963; Botero-Velez et al., 1994). Clinical studies revealed that affected patients did not respond to the mineralocorticoid antagonist, spironolactone, but that a low salt diet plus triamterene, a specific ENaC blocker, did correct the hypertension. Interestingly, all of the identified Liddle’s syndrome mutations affected the C-terminal domains of β and γ ENaC, leading to either deletion or modification of the functional important proline-rich regions (Shimkets et al., 1994; Hansson et al., 1995). Expression of truncated β or γ ENaC in *Xenopus laevis* oocytes led to an increase in amiloride-sensitive Na+ transport compared to intact β or γ ENaC (Schild et al., 1995; Snijder et al., 1995; Firsov et al., 1996). These truncations did not alter the single-channel conductance or open state probability, suggesting an increase in the number of active channels in the plasma.
membrane. As described previously in this introduction, the proline-rich motifs of α, β and γ ENaC can interact with the WW-domains of the binding protein Nedd4. Deletion or modifying this proline-rich region would, therefore, prevent correct Nedd4 binding and channel degradation, with consequent increased ENaC channel numbers in the plasma membrane.

Pseudohypoaldosteronism

Autosomal recessive mutations of α, β, γ ENaC, cause pseudohypoaldosteronism type 1 (PHA1), a salt wasting syndrome. This syndrome is characterized by hypotension, mineralocorticoid-resistance with hyperkalemic acidosis, and high plasma aldosterone levels (Hanukoglu et al., 1994; Chang et al., 1996). PHA1 patients are therefore treated with sodium and bicarbonate supplementation. In several families showing this form of PHA1 mutations in all of the three ENaC subunit genes were found (Chang et al., 1996; Strautnieks et al., 1996; Gründer et al., 1997). In contrast to the gain of function mutations in Liddle’s syndrome, loss of function mutations can, in principle, operate on any mechanism disturbing normal channel function. Therefore, in PHA1, the mutations are more heterogeneous, and result in either amino acid substitutions, splice variants or premature truncations. *Xenopus laevis* oocytes expressing PHA1-mutant channels showed reduction in ENaC activity compared to oocytes expressing wild-type ENaC subunits (Chang et al.1996).

Aim and outline of this thesis

The general aim of this thesis was to study the regulation of Na⁺ reabsorption by aldosterone in mammalian CNT/CCD cells, and the regulation and oligomerization of ENaC in particular. The functional aspects of ENaC have been studied mainly by heterologous ENaC expression in the nonepithelial *Xenopus laevis* oocyte system and the amphibian A6 cell line. In this thesis, we used a mammalian CNT/CCD cell model. To this end, rabbit CNT/CCD cells were isolated by immunodissection and cultured on permeable filters. This model system has retained several characteristics of the original epithelium, including
lumen-negative transepithelial potential differences, responsiveness to aldosterone and benzamil sensitivity.

In Chapter 2, we characterized the aldosterone action in this mammalian CNT/CCD model system and compared it with the aldosterone response in amphibian tissue (A6 cell line). The cloning of the three ENaC subunits $\alpha$, $\beta$ and $\gamma$ ENaC made it possible to relate effects on Na$^+$ reabsorption to the ENaC mRNA and protein levels.

Little is know about the mechanism by which aldosterone stimulates Na$^+$ reabsorption. Therefore, in this thesis we studied the regulation of ENaC and the aldosterone responsiveness of its subunits. In Chapter 3, we determined whether the rate of Na$^+$ influx modulates the effect of aldosterone on transepithelial Na$^+$ transport and ENaC expression.

To identify the pathways of Na$^+$ fluxes and intracellular Na$^+$ homeostasis in more detail, in Chapter 4 quantitative fluorescence microscopy was used in combination with the sodium-sensitive probe SBFI. We measured the intracellular Na$^+$ concentrations in individual cells at various transport conditions.

ENaC is formed by heteromultimerization of $\alpha$, $\beta$ and $\gamma$ ENaC subunits. However, the number of subunits and the stoichiometry of the ENaC complex is still a matter of debate. In Chapter 5, we determined the oligomerization state of ENaC when overexpressed in the Xenopus laevis oocyte expression system.

In Chapter 6, the results were summarized and a general discussion with future perspectives was given.

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WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na⁺ channel deleted in Liddle’s syndrome. EMBO J. 15, 2317-2380


CHAPTER 2

Time-dependent regulation by aldosterone of the amiloride-sensitive Na\(^+\) channel in rabbit kidney.

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Summary

The epithelial Na+ channel (ENaC) functions as the rate-limiting step in aldosterone-regulated transcellular Na+ transport. In this study, the effect of aldosterone on ENaC mRNA levels, protein synthesis and benzamil-sensitive Na+ transport was investigated using primary cultures of immunodissected rabbit connecting tubule and cortical collecting duct cells (CNT and CCD). After a lag time of 3 h aldosterone increased transepithelial Na+ transport to a maximal level of 260 ± 44%, which value was reached after 16 h of incubation. The α, β and γ ENaC mRNA levels measured by semi-quantitative RT-PCR, were not changed by aldosterone during the first 3 h but a twofold increase was apparent after 6 h and remained elevated up to 16 h of incubation. Immunoprecipitation of [35S]methionine labeled ENaC revealed a rise in protein level of the α and β subunits, but the protein level of the γ subunit remained constant. In conclusion, our data suggests that in rabbit CNT and CCD the early increase of Na+ transport by aldosterone could be due to activation or insertion of existing Na+ channels into the apical membrane and that the late response is mediated by increased synthesis of the α and β ENaC subunits.

Introduction

The epithelial Na+ channel (ENaC) plays an important role in Na+ homeostasis and, consequently, in the long-term regulation of blood pressure [14]. ENaC is located in the apical membrane of epithelial cells in the distal part of the nephron, urinary bladder, distal colon, salivary and sweat ducts, respiratory tract and taste buds [15]. This Na+ channel consists of three subunits α, β and γ ENaC, which share at amino acid level ~36% identity [5]. Each subunit has two transmembrane domains with a large extracellular loop and short cytoplasmic N and C termini [18]. Two possible assemblies of ENaC have been proposed recently: a four-subunit stoichiometry of 2α,1β,1γ indicated by Firsov et al. [10] and a nine-subunit stoichiometry of 3α,3β,3γ suggested by Snijder et al. [21]. The physiological significance of ENaC has been demonstrated by gain of function mutations in the individual subunits forming ENaC, which cause Liddle’s syndrome, an inherited form of salt-sensitive
hypertension [20, 13]. Alternatively, in pseudohypoaldosteronism missense mutations in the
$\alpha$ and $\beta$ subunits result in loss of ENaC function [6].

The steroid hormone aldosterone plays a major role in the regulation of ENaC but the
mechanisms involved are not completely understood [12, 19]. There are three potential
mechanisms of aldosterone action. First, induction of synthesis or insertion of $\text{Na}^+$ channels
into the apical membrane. Second, stimulation of inactive channels already present in the
plasma membrane. Third, increasing the open probability of the channels [11]. Previous
studies in amphibian tissues including A6 cells and frog skin, have established that the
aldosterone response is composed of distinct phases. After a latent period of 30-90 min, an
early response is apparent during which $\text{Na}^+$ transport increases and transepithelial electrical
resistance falls, and finally a late response from 3 to 24 h, during which $\text{Na}^+$ transport further
increases [11, 16]. In A6 cells, aldosterone induced accumulation of $\alpha$, $\beta$ and $\gamma$ ENaC
mRNA after 24 h. However, the effect of aldosterone on the rate of protein synthesis was not
related to the effect on mRNA abundance. As early as 1 h after aldosterone addition, the rate
of synthesis of the $\alpha$ subunit was significantly increased. The effect on the rate of synthesis
of the $\beta$ subunit became significant after 6 h hormone addition, whereas the effect on the $\gamma$
subunit did not reach significance [16].

The aim of the present study was to examine the regulation of ENaC by aldosterone in
mammalian kidney cells. To this end, primary cultures of rabbit connecting tubule and
cortical collecting duct cells (CNT and CCD) were used. This model system retains several
characteristics of the original epithelium including a benzamil-sensitive lumen-negative
transepithelial potential difference [4]. In the study presented here, we examined the time
dependent effect of aldosterone on benzamil-sensitive $\text{Na}^+$ transport, mRNA and protein
level for each of the three rbENaC subunits. Aldosterone-induced $\text{Na}^+$ transport was
measured in an Ussing chamber set up as the benzamil-sensitive short-circuit current. To
determine the effect of aldosterone on rbENaC mRNA levels a radioactive RT-PCR was
developed. The effect of aldosterone on protein synthesis was analysed by
immunoprecipitation of [$^{35}$S]methionine labeled rabbit connecting tubule and cortical
collecting duct cells.
Materials and methods

- **Primary cultures of rabbit kidney cortical collecting system**
  Rabbit kidney connecting tubule (CNT) and cortical collecting duct (CCD) cells were immunodissected from kidney cortex of New Zealand white rabbits (~0.5 kg body wt) with the monoclonal antibody R2G9 and set in primary culture on permeable filters (0.33 or 4.7 cm²; Costar, Cambridge, MA, USA) as previously described in detail [3]. All experiments were performed with confluent monolayers between 5 and 8 days after seeding the cells. Transepithelial potential difference was routinely checked before every experiment, to confirm confluence and intactness of the monolayer.

- **Ussing chamber experiments**
  For measuring transcellular short-circuit current (Isc), filter cups (area 0.33 cm²) were mounted between two half chambers and bathed at 37°C with incubation medium containing (in mM): 140 NaCl, 2 KCl, 1 K₂HPO₄, 1 KH₂PO₄, 1 MgCl₂, 1 CaCl₂, 5 glucose, 5 L-alanine, 10 Hepes-Tris (pH 7.4). The solutions bathing the monolayers were connected via agar bridges and Ag-AgCl electrodes to a voltage-clamp current amplifier (Physiological Instruments, San Diego, CA, USA) and Isc was recorded before and after apical addition of 10⁻⁵ M benzamil. The benzamil-sensitive component of Isc was used as an estimate of transcellular Na⁺ transport. The filters were placed 30 min before the end of the aldosterone incubation period in the Ussing chamber set up in the presence or absence of 10⁻⁷ M aldosterone in the medium.

- **Screening of rabbit CCD and CNT cDNA library and designing of PCR primers**
  XbaI/HindIII, HincII and HincII cDNA fragments, containing the regions 970 to 2782 bp, 4 to 2217 bp and 4 to 2036 bp of the rat α, β and γ ENaC, respectively, were random primed with [α²³⁵P]dCTP (Amersham, Pharmacia Biotech, Uppsala, Sweden), and were used to screen a rabbit CCD and CNT cDNA library. The size selected cDNA library (1.5-4.0 kb) was obtained by Moloney murine leukemia virus reverse transcriptase (Mmlv RT) superscript II (Gibco, Paisley, UK) of rabbit CCD and CNT RNA and cloned into pSport
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plasmid vector (Gibco). The hybridization conditions were: 250 mM Na₂HPO₄/NaH₂PO₄ (pH 7.2), 7% (wt/vol) SDS, 1 mM EDTA, at 55°C O/N, while the final washing was performed in 100 mM Na₂HPO₄/NaH₂PO₄ (pH 7.2), 0.1% (wt/vol) SDS, 1 mM EDTA for 30 min at 55°C. After two rounds of rescreening, a positive clone for each α, β and γ rbENaC fragment was isolated. The reading frame of the fragments α, β and γ rbENaC started in the extracellular domain at AA number 218, 244 and 498, respectively. Sense and antisense primers were designed to perform a polymerase chain reaction (PCR). The primers for the three rbENaC subunits were as follows: the α upper 5’ TGG CGA GGA AAG ACT GG, the α lower 5’ TCA TCC TGT CCG TGC AC, the β upper 5’ CTG AAG CTG ATC CTG GAC, the β lower 5’ CAA TGA TGA TCT CGG CAA AC, the γ upper 5’ CGA GAT GCT TCT GTC CCA AT, and the γ lower 5’ CAG GTC GTC GTC GTC TAT CTC. The α, β and γ primer sets bracket the sequence from 738 to 1156 bp, 861 to 1608 bp, and 1575 to 1809 bp of α, β and γ rbENaC, respectively.

• **RT-PCR**

Total RNA was isolated by guanidinium isothiocyanate/phenol-chloroform extraction from the CCD and CNT cells cultured on permeable filters (4.7 cm²) in the presence or absence of aldosterone [7]. cDNA was synthesized in 30 μl RT-mix containing 1 μg RNA and 200 U RNA Mmlv RT (Gibco). PCR was performed on serial dilutions of this RT-mix in PCR buffer (100 μM dNTP’s, 1 U Taq polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% (wt/vol) gelatin, 3.0, 2.0 and 2.5 mM MgCl₂ for the α, β and γ rbENaC primers, respectively, 1 μCi [α³²P]dCTP and the designed primer sets in a total volume of 50 μl).

The β-actin mRNA level was, as internal control, determined in the same PCR reaction, using the primers corresponding to the coding region of the human β-actin cDNA sequence. The β-actin upper and lower primer were: 5’ GCT ACG AGC TGC CTG ACG G and 5’ GAG GCC AGG ATG GAG CC, respectively, and bracket the sequence from 756 to 1084 bp of the human β-actin. The amounts of the rbENaC primers in combination with the β-actin primers were for the α and β-actin 50 and 20 ng, for the β and β-actin 50 and 15 ng and for the γ and β-actin 55 and 20 ng. The thermal cycling programs were as follows: 94°C
for 1 min, 58°C (α, β) or 54°C (γ) for 1 min and 72°C for 1 min. After 35 cycles of amplification, the samples were fractionated on a 5% (vol/vol) polyacrylamide gel. The amount of [α-32P]dCTP incorporation was determined with a Phosphor Imager (Biorad, Hercules, CA, USA) using the programme Molecular Analyst (Biorad).

• **Generation of ENaC antibodies**

Antibodies were raised against the α rENaC domain AA 269-471 (α rENaC:Sau3A), β rβENaC AA domain 244-510 (β rβENaC:HinDIII/KPN) and γ rγENaC domain AA 173-510 (γ rγENaC:BamHI). These domains were cloned into pGEX-3X (Pharmacia), pQE-32 (Qiangen, Hilden, Germany) and pGEX-2T (Pharmacia), respectively. The constructed plasmids were transformed into DH5α and protein expression was induced for 2 h with IPTG (0.1 M). The pellet fraction of the bacteria, containing the fusion proteins, was isolated from a 10% (vol/vol) polyacrylamide gel. The concentration of the isolated proteins was measured with Biorad protein assay dye reagent concentrate. The glutation S-transferase (α and γ ENaC) and histidine-tagged (β ENaC) fusion proteins were used to immunize guinea pigs. The resulting antisera were affinity purified by column chromatography.

• **Immunohistochemistry**

Kidneys of New Zealand white rabbits fed a low Na+ diet were perfused for 3 min with 0.1 M phosphate buffered saline (PBS: 137 mM NaCl, 8.4 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl) containing 5000 U/l heparine, 4 mM procaine, 75 mM L-lysine-monohydrochloride, then subsequently perfused for 15 min with 1% (vol/vol) periodate lysine paraformaldehyde fixative (PLP) containing 100 mM NaCl, and finally incubated for 2 h with 1% (wt/vol) PLP. The perfused kidneys were cut into pieces, incubated for 2 h in 1% (wt/vol) PLP at RT, subsequently incubated in 20% (wt/vol) sucrose in PBS O/N at 4°C, frozen in liquid nitrogen and finally stored at -80°C. Sections of 7 μm were incubated for 30 min with 0.3% (vol/vol) H2O2 in PBS, washed three times for 5 min in TN (0.1 M Tris/HCl, pH 7.5, 0.15 M NaCl), and then incubated for 10 min with 0.1% (wt/vol) SDS in TN (for α ENaC) or for 10 min with 0.2% (vol/vol) Triton X-100 in TN (for β and γ ENaC). The sections were washed three times for 5 min with TN, blocked for 30 min with TNB (TN and
0.5% (vol/vol) blocking reagent (NEN life science products, Boston, MA, USA), washed three times for 5 min with TNT (TN and 0.05% (vol/vol) tween 20), and finally incubated O/N at 4°C with a 1:500 dilution of guinea pig affinity-purified polyclonal anti α, β or γ ENaC. Subsequently, sections were washed three times for 5 min with TNT and incubated for 1 h at RT with anti-guinea pig biotine 1:2000 (vol/vol) in TNB. The sections were washed three times for 5 min in TNT, incubated for 45 min at RT with SA-HRP 1:100 (vol/vol) in TN, washed three times for 5 min with TNT and incubated for 7 min at RT with fluorophore Tyramide in amplification diulent (NEN). The sections were washed three times for 5 min with TNT, incubated for 30 min at RT with R2G9 hybridoma supernatant to double stain the sections and finally washed three times for 5 min with TNT. Next incubated for 1 h at RT with a 1:50 dilution of TRITC-conjugated goat anti-mouse IgG (Sigma, St.Louis, MO, USA) in TNB. The sections were washed three times, dehydrated in 50-100% (vol/vol) methanol and mounted in mowiol 4-88 (Hoechst, Frankfurt-am-Main, Germany) containing 2.5% (wt/vol) NaN₃. Photographs were taken with a Zeiss Axioskop microscope equipped with epifluorescent illumination and Kodak EPH P1600X films were used.

**Immunoprecipitation**

CCD and CNT cells cultured on permeable filters (4.7 cm²) in the presence or absence of aldosterone were washed three times for 5 min with DMEM without methionine (Biowhittaker, Walkersville, MD, USA). The monolayers were labeled at 37°C for 2 h by apical addition of 2.5 mCi/filter trans [³⁵S]methionine (ICN Pharmaceuticals, Irivine, CA), subsequently washed at 4°C with 25 ml KHB (in mM: 128 NaCl, 5 KCl, 1.2 MgSO₄, 10 Na-acetate, 2 NaH₂PO₄, 10 glucose, 20 Hepes, 1 L-alanine, 4 L-lactate, 1 CaCl₂) and scraped in 275 μl homogenization buffer (20 mM Tris-HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 6 mM β-mercapto-ethanol, 10 mM phenylmethylsulfonylfluoride, 5 mg/ml leupeptine and 5 mg/ml pepstatin). Subsequently, the cells were sonicated three times for 30 s and the [³⁵S] incorporation was determined by liquid scintillation. Samples containing equal amounts of counts (6.10⁶ cpm) were deglycosylated and/or immunoprecipitated. Deglycosylation was realized with 1500 U/sample PNGase F according to the protocol described by manufacturer (New England Biolabs, UK). For immunoprecipitation, SDS was added to control as well as
to deglycosylated samples to a final concentration of 3.6% (wt/vol), and samples were incubated for 5 min at 95°C. Next, the samples were incubated O/N at 4°C with the affinity purified α, β or γ ENaC antisera in TENT 1% (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% (vol/vol) Triton X-100). These samples were incubated with prewashed protein A-sepharose beads (Kem-En Tec, Copenhagen, Denmark) for 2 h at RT and the beads were subsequently washed five times with 1% (vol/vol) TENT and three times with NET 2 (10 mM Tris-HCl, pH 7.5, 0.1% (wt/vol) SDS, 2 mM EDTA). The immunoprecipitated proteins were resuspended in 25 μl Laemmli sample buffer, and incubated for 5 min at 95°C. Next, 20 μl of the samples were loaded on 7% (wt/vol) SDS-polyacrylamide gel and electrophoresed. The gel was stained for 10 min at 65°C with 0.25% (wt/vol) Coomassie Brilliant blue, 10% (vol/vol) acetic acid, 50% (vol/vol) methanol, destained two times for 10 min at 65°C with 7% (vol/vol) acetic acid, 25% (vol/vol) methanol, rinsed in water. To enhanced the signals, the gel was incubated two times for 10 min with DMSO, and two times for 15 min with 20% (wt/vol) 2,5-diphenyloxazole (Sigma) in DMSO. After two times for 5 min rinsing in distilled water, the gel was dried, and exposed to a film with an intensifying screen at -80°C. Quantification of [35S] incorporation was determined by densitometry using a line scanner (Mirror Scanner, Edina) and an image-analysis Macintosh program (NIH Image).

- **Chemicals**

Benzamil was obtained from Research Biochemical International (Natick, MA, USA). Taq polymerase was isolated as described by Engelke et al. (9). PNGase F was obtained from New England Biolabs. All other chemicals were obtained from Sigma. Benzamil and aldosterone were dissolved in ethanol. Final concentrations of solvent never exceeded 0.1% (vol/vol). Identical vehicle concentrations were used as a control. Aldosterone was added to the apical and basolateral side, as described in the text.

- **Statistics**

Results are given as means ± SEM. For Ussing chamber experiments and RNA isolations statistical significance was determined by analysis of variance (ANOVA) and in case of
significance individual groups were compared by contrast analysis according to Fisher. RT-PCR experiments were analyzed by linear regression and the calculated slopes were compared by paired Student’s t-test. Data of the immunoprecipitations were compared using paired Student’s t-test. P<0.05 was considered statistically significant.

Results

Effect of aldosterone on benzamil-sensitive short-circuit current ($I_{sc}$)

The primary cultures of the rabbit connecting tubules (CNT) and cortical collecting duct (CCD) cells respond to aldosterone with a time-dependent increase in transepithelial Na$^+$ transport measured as the benzamil-sensitive short-circuit current ($I_{sc}$). After 3, 6, and 16 h of incubation with aldosterone ($10^{-7}$ M, both sides), the benzamil-sensitive $I_{sc}$ was significantly (P<0.05) increased when compared to non-stimulated monolayers, whereas after 2 h no significant increase (P>0.1) was apparent (Fig. 1).

Effect of aldosterone on rbENaC mRNA levels

Preliminary studies using Northern blot analysis indicated very low to undetectable levels of ENaC mRNA in the rabbit primary cultures. Therefore, we developed a semi-
quantitative RT-PCR assay to determine the effect of aldosterone on rbENaC mRNA levels. First, four nondegenerate sense and antisense primer sets were designed corresponding to α, β and γ rbENaC and human β-actin sequences. RT-PCR amplification performed on total RNA extracted from the primary cultures revealed single fragments of 419 bp, 746 bp, 234 bp and 328 bp, respectively (Fig.2).

![Figure 2: Detection of α, β and γ rbENaC mRNA ethidium-bromide stained agarose gel (2% wt/vol) using reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR was performed with primers for α rbENaC (lane 2-5), β rb ENaC (lane 6-9) and γ rbENaC (lane 10-13). As template was used 0.1 µg RNA isolated from primary cultures of rabbit CCD and CNT (lane 1-3, 6, 7, 10 and 11), cDNA clone of β-actin and α, β or γ rb ENaC fragments (lane 4, 8, 12) or water (lane 5, 9, 13). As second set of primers, β-actin primers were used in lane 3-5, 7-9 and 11-13. In lane 1, RT-PCR was performed with the β-actin primers only.

These PCR products were of the expected size based on the published sequences. In subsequent PCR analysis the subunit specific rbENaC primers were combined with β-actin as an internal control which produced the same fragments as in their respective individual assays. Total RNA of 0, 2, 3, 6 and 16 h aldosterone-treated primary cultures of CNT and CCD cells (10^-7 M, both sides) were used as template to determine rbENaC mRNA levels by radioactive RT-PCR. The yield of total RNA was 17 ± 2 µg/filter, with no significant
differences among the specimen. To normalize the amount of mRNA present in the individual PCR assay, β-actin was co-amplified as internal control. Results of a typical ENaC RT-PCR analysis, using three serial dilutions of cDNA obtained from 16 h aldosterone-treated and control monolayers, are shown in figure 3.

![Figure 3: Representative RT-PCR of α rbENaC (A), β rbENaC (B) and γ rbENaC (C) using serial dilutions of RNA isolated from control and 16 h aldosterone (10^{-7} M, both sides) treated primary cultures of CCD and CNT. In all reactions β-actin was used as internal control. Upper figures depict the RT-PCR products as a function of the DNA concentration and lower figures illustrate quantitative analysis of the incorporated [α-32P]dCTP and the corresponding line was calculated by linear regression. The relative amounts of rbENaC mRNA were expressed as the ratio of the rbENaC slope and the matching β-actin slope. 100% cDNA is equivalent with 17 ng RNA.](image)

Incorporation of [α-32P]dCTP from the individual PCR-products as a function of initial cDNA concentration was quantified by densitometry and the corresponding slope was calculated by linear regression. The relative amounts of α, β and γ rbENaC mRNA were expressed as the ratio of the α, β and γ rbENaC slope respectively, and the matching β-actin slope. The mean values calculated from six independent experiments are presented in figure 4. After 2 and 3 h aldosterone treatment α and β rbENaC mRNA levels started to rise, whereas after 6 and 16 h aldosterone incubation these levels were maximally increased to
approximately 160% (P<0.05). The aldosterone effect on γ rbENaC mRNA accumulation exhibited a slower time course since an increase was only apparent after 6 and 16 h of aldosterone treatment. At these two latter time points a similar maximal level as with the α and β rbENaC was reached.

Figure 4: Time dependent effect of aldosterone on the expression of rbENaC mRNA. Levels of α, β and γ rbENaC mRNA were determined by RT-PCR, using RNA isolated from control and aldosterone (10^{-7} M; 2, 3, 6 and 16 h) treated rabbit primary cultures of CNT/CCD. Aldosterone-induced mRNA levels were calculated as described in the legend of figure 3 and normalized for the corresponding control mRNA levels. Data are means ± SEM of 6 experiments. *, significant different from control (P<0.05)

Immunolocalization of ENaC in rabbit kidney

Affinity purified antibodies raised against the α, β and γ ENaC subunits were used for immunohistochemistry on sections of rabbit kidney cortex. The rabbits were kept for two weeks on a low Na^{+} diet, which dramatically reduced the urinary Na^{+} excretion from a concentration of 1.6 mmol Na^{+} to an undetectable level. Immunopositive staining for α, β and γ ENaC colocalized in connecting tubules and collecting ducts in the cortex as well as in the outerstripe of the outer medulla (Fig.5).
The immunopositive staining was confined to the apical domain. Importantly, antibody R2G9, which recognizes a cell surface antigen specific for rabbit connecting tubules and collecting ducts, colocalized with ENaC and this antibody is routinely used to immunodissect connecting tubules and cortical collecting ducts from rabbit kidney [3]. Of note, to unmask the epitope of the α subunit it was necessary to pretreat the sections with 0.1% (wt/vol) SDS. After this treatment only a minority of the distal tubules were immunopositive, suggesting that this epitope is nearly inaccessible. No staining was observed in glomeruli, proximal and distal convoluted tubules. In the thick ascending limb of Henle’s loop a faint cytoplasmic staining of the α subunit was apparent, but at present the significance of this finding is unclear.

**Effect of aldosterone on rbENaC protein levels**

The α, β and γ ENaC antibodies immunoprecipitated proteins of ~90, ~97 and ~89 kD, respectively, from primary cultures of rabbit CCD and CNT cells (Fig.6). After
deglycosylation the MW of the rbENaC subunits decreased to ~66, ~82, and ~76 kD, respectively.

A:

![Immunoblot showing α rbENaC, β rbENaC, and γ rbENaC before and after PNGase F treatment](image)

B:

![Representative time course of aldosterone effect on α rbENaC, β rbENaC, and γ rbENaC protein levels](image)

**Figure 6:** Immunoprecipitation of rbENaC subunits of primary cultures of CCD and CNT. A) Immunoblots showing α rbENaC (lane 1 and 2), β rbENaC (lane 3 and 4) and γ rbENaC (lane 5 and 6), before (lane 1, 3 and 5) and after PNGase F treatment (lane 2, 4 and 6). Primary cultures were pulse labeled with [35S]methionine for 2 h. Molecular weight standard is denoted on the left. B) Representative time course of aldosterone (10^-7 M) effect on α rb ENaC (upper), β rb ENaC (middle) and γ rbENaC (lower) protein level. During the last two hours of the stimulation period, cells were labeled with [35S]methionine.
The deglycosylated α rbENaC subunit appeared to be smaller than can be deduced from its sequence [5]. To test whether this reduction is due to an effect of endogenous serine-protease activity, the monolayers were exposed to aprotinin, a serine-protease inhibitor [22]. Overnight incubation in the presence of aprotinin (28 μg/ml) did not affect the size of the α rbENaC subunit (data not shown). Of note, immunoprecipitation with the guinea pig antibody against γ ENaC resulted in two distinct bands. Since only the lower band was observed when a rabbit antibody against the γ ENaC was used, protein quantification was restricted to this lower band. No cross-reactivity was observed between the antibody against a particular ENaC subunit and the two other rbENaC subunits. Figure 7 shows the time-dependent effect of aldosterone (10⁻⁷ M, both sides) on the three rbENaC subunit protein levels of CCD and CNT cells cultured in the absence or presence of aldosterone. Aldosterone only stimulated the α and β rbENaC protein synthesis after 6 and 16 h (P<0.05), while γ rbENaC subunit protein levels remained unaffected during the entire incubation period.

![Figure 7: Time dependent effect of aldosterone on protein synthesis of α, β and γ rbENaC. Levels of α, β and γ rbENaC were determined by immunoprecipitation of [³⁵S]methionine labeled primary cultures of CCD and CNT cells in the absence of presence of aldosterone. Monolayers were incubated with 10⁻⁷ M aldosterone for 2, 3, 6 or 16h. Values were normalized for the corresponding control protein level. Data are means ± SEM of 6 experiments. *, significant different from control (P<0.05).]
Discussion

The present study reports for the first time the time-dependent effect of aldosterone on ENaC mRNA accumulation, protein synthesis and benzamil-sensitive Na⁺ transport in a mammalian model system consisting of primary cultures of rabbit connecting tubule and cortical collecting duct cells. After a lag time of 3 h, aldosterone stimulated transcellular electrogenic Na⁺ transport in a time-dependent manner. This stimulation of Na⁺ current was clearly dissociated from a 3 h later observed increase in α, β rbENaC mRNA and protein levels, while for γ ENaC only the mRNA level was upregulated.

There are three possible mechanisms for aldosterone stimulated Na⁺ transport: firstly, the synthesis and insertion of ENaC subunits; secondly, the activation of existing Na⁺ channels by regulatory factors, the so-called aldosterone-induced proteins (AIP’s); and thirdly, insertion of Na⁺ channels by AIP’s [23]. This study shows that after 3 h of aldosterone treatment the benzamil-sensitive $I_{sc}$ was increased whereas at the same time rbENaC mRNA and protein levels were not significant stimulated. This indicates that the early increase of aldosterone-induced Na⁺ transport is mediated, at least in part, by aldosterone induced regulatory factors.

A further increase of the benzamil-sensitive $I_{sc}$ to maximal levels occurred between 6 and 16 h of aldosterone incubation, and this stimulation was accompanied by a twofold increase in α and β rbENaC protein levels. A surprising finding is that γ ENaC protein does not increase after aldosterone exposure suggesting that γ subunit synthesis is not necessary for aldosterone to stimulate Na⁺ transport. Since only the combination α, β and γ subunits of ENaC results in a maximal expression of the Na⁺ channel [5], we anticipate an excess of γ rbENaC in the primary cultured cells. Consequently, the late response to aldosterone is due to increased synthesis of α and β ENaC and subsequent insertion of α, β and γ ENaC complexes. An alternative explanation is that the oligomeric composition of the ENaC complex is changed during hormonal stimulation, as proposed by Asher et al. [1]. This latter suggestion could mean that during aldosterone stimulation of Na⁺ transport, the α and β subunit stoichiometry relative to the γ subunit is increased in ENaC oligomers. The subunit assembly of ENaC is still a matter of debate since 2β, 1β and 1γ composition has been
postulated [10], while others suggested a nine subunit complex consisting of 3α, 3β and 3γ subunits [21]. Further studies are, therefore, warranted to address the ENaC stoichiometry in different physiological situations.

Similar augmented levels of mRNA for each rbENaC subunit were reached after 6 h of aldosterone incubation. Assuming that the rate of degradation of the subunits is not changed by aldosterone as described for A6 cells [16] together with the present finding that the relative increase in transcription is lower than the relative increase in translation, suggests that α and β rbENaC are upregulated by both the transcription and translation machinery. Surprisingly, the stimulated transcription of γ rbENaC did not result in increased synthesis of this subunit. This could mean that the translation of γ rbENaC mRNA is already taking place at maximal rate or that the translation rate is decreased. At present it is unclear which mechanism is responsible for the observed effect.

In primary cultures of CNT and CCD cells, aldosterone stimulated Na⁺ transport significantly after a lag time of 3 h. In contrast, in A6 cells, originally derived from *Xenopus laevis*, aldosterone enhanced Na⁺ transport as early as 1 h after addition. A similar discrepancy was observed at the protein level, since in A6 cells the α ENaC subunit was upregulated after 1 h, whereas in mammalian primary cultures ENaC protein levels remained at control levels during the first hours of aldosterone addition [16]. The absence of these early effects in the rabbit cultures could be due to several circumstances. For instance, differences in the baseline levels of Na⁺ transport between both studies could be responsible for the relative importance of the early or late response of epithelial cells to aldosterone [23]. In addition, in mammalian and amphibian epithelia aldosterone-induced Na⁺ transport could be regulated in a different manner.

It is of interest to note that in different target organs like lung, colon and kidney, ENaC expression is regulated differently by aldosterone. Increase of ENaC activity in rat lung is, especially around birth, positively controlled by glucocorticoid receptors mediated by stimulation of the transcription of all three rENaC subunits, whereas in rat colonic cells, the level of α rENaC messenger remained constant during aldosterone exposure, while a major stimulatory effect is observed on β and γ rENaC mRNA accumulation [2, 15]. These effects are different from those observed in rat kidney, where aldosterone failed to alter rENaC
mRNA levels [17] or slightly increased α rENaC mRNA while β and γ rENaC remained constant [1]. These latter findings are, however, in contrast to our results and data presented by Denault et al. [8]. Since these previous studies examined by Northern blot analysis mRNA levels in whole kidney or in kidney cortex, respectively, a modest increase in mRNA abundance could have remained undetected.

References


Modulation of the aldosterone-induced stimulation of ENaC synthesis by changing the rate of apical Na\textsuperscript{+} entry.

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Summary

Primary cultures of immunodissected rabbit connecting tubule and cortical collecting duct cells were used to investigate the effect of apical Na⁺ entry rate on aldosterone-induced transepithelial Na⁺ transport, which was measured as benzamil-sensitive short-circuit current (Iₛₑ). Stimulation of the apical Na⁺ entry, by long-term short-circuiting the monolayers, suppressed the aldosterone-stimulated benzamil-sensitive Iₛₑ from 320 ± 49% to 117 ± 14%, while in the presence of benzamil this inhibitory effect was not observed (335 ± 74%). Immunoprecipitation of [³⁵S]methionine-labeled β rbENaC revealed that the effects of modulation of apical Na⁺ entry on transepithelial Na⁺ transport are exactly mirrored by β rbENaC protein levels, since short-circuiting the monolayers decreased aldosterone-induced β rbENaC protein synthesis from 310 ± 51% to 56 ± 17%. Exposure to benzamil doubled the β rbENaC protein level to 281 ± 68% in control cells, but had no significant effect on aldosterone-stimulated β rbENaC levels (282 ± 68%). In conclusion, stimulation of apical Na⁺ entry suppresses the aldosterone-induced increase in transepithelial Na⁺ transport. This negative feedback inhibition is reflected in a decrease in β rbENaC synthesis or in an increase in β rbENaC degradation.

Introduction

The mineralocorticoid hormone aldosterone plays a major role in Na⁺ homeostasis and, consequently, in extracellular volume regulation by controlling epithelial Na⁺ channel (ENaC) expression in the kidney (14). The ENaC complex, consisting of three subunits α, β and γ ENaC, is present in the apical membrane of epithelial cells in the distal kidney, distal colon, salivary glands, sweat glands, respiratory tract and taste buds (3, 23). It has been demonstrated that upregulation of ENaC expression by aldosterone differs among mammals (23), and is also tissue-specific (30, 32).

There are three possible mechanisms for aldosterone to enhance Na⁺ transport: first, the synthesis and insertion of ENaC subunits into the apical membrane; second, the activation of existing Na⁺ channels by regulatory proteins, by so called aldosterone-induced proteins (AIP’s); and third, the increase of the open probability of Na⁺ channels (34). In primary
cultures of rabbit kidney connecting tubule and cortical collecting duct cells (CNT and CCD), the first phase of aldosterone-stimulated transepithelial Na⁺ transport is likely to be mediated by AIP’s. During the late phase of aldosterone action, the three fold increase in apical Na⁺ transport is accompanied by an increase in rbENaC mRNA for all three subunits, but with only higher α and β subunit protein levels (5).

In addition to regulation of ENaC by aldosterone (5, 24, 25), long-term exposure to vasopressin also stimulates ENaC expression (8). Several other studies identified additional mechanisms involved in the regulation of ENaC activity, including changes in pH, ATP, Ca²⁺ concentrations, Na⁺ concentrations and cell swelling (10, 13, 19). Notably, the role of these parameters was studied on the short-term action of ENaC. Furthermore, the role of these factors in aldosterone-induced stimulation of transepithelial Na⁺ transport has not been investigated. The aim of the present study was, therefore, to investigate the effect of the rate of apical Na⁺ entry on long-term effects of aldosterone. So far, the mechanisms by which ENaC synthesis is regulated in response to changes in the rate of apical Na⁺ entry are still poorly understood. Two forms of negative feedback regulation by increased Na⁺ concentrations have been described (1, 33) namely, self inhibition and feedback inhibition. Self inhibition could be due to a direct interaction of extracellular Na⁺ with ENaC itself (28). In salivary duct cells it is found that Na⁺ channel activity does not change with increasing extracellular Na⁺ (19), whereas in frog skin the Na⁺ channel activity is controlled by extracellular Na⁺ (11). On the other hand, feedback inhibition could also be mediated by an increase in the intracellular Na⁺ concentration (19).

Primary cultures of rabbit CNT and CCD were used to study the effect of changes in driving force for apical Na⁺ entry on aldosterone regulation of ENaC activity. In one protocol primary cultures were short-circuited to stimulate apical Na⁺ influx. In another protocol monolayers were incubated overnight with benzamil to block apical Na⁺ influx. In both protocols benzamil-sensitive short-circuit current (Iₛᵣ) and β rbENaC protein levels were determined.
Materials and methods

• **Primary cultures of rabbit kidney cortical collecting system**
Rabbit kidney connecting tubule and cortical collecting duct cells (CNT and CCD), hereafter referred to as the cortical collecting system, were immunodissected from kidney cortex of New Zealand white rabbits (± 0.5 kg body wt) with the monoclonal antibody R2G9 and set in primary culture on permeable filters (0.33 or 1.13 cm²; Costar, Cambridge, MA, USA) as previously described in detail (2). All experiments were performed with confluent monolayers between 5 and 8 days after seeding the cells. 16 h prior to the experiments, the monolayers were short-circuited by flooding the monolayers with culture medium, incubated with aldosterone (10⁻⁷ M, both sides) or benzamil (10⁻⁵ M, apical) and combinations of these treatments.

• **Ussing chamber experiments**
For the measurement of transepithelial short-circuit current (I_sc), filter cups (area 0.33 cm²) were routinely washed 3 times with incubation medium containing: 140 mM NaCl, 2 mM KCl, 1 mM KH₂PO₄, 1 mM KH₂PO₄, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, 5 mM l-alanine, 10 mM Hepes-Tris (pH 7.4) and then mounted between two half chambers and bathed at 37°C with incubation medium. The solutions bathing the monolayers were connected via agar bridges and Ag-AgCl electrodes to a voltage-clamp current amplifier (Physiological Instruments, San Diego, CA, USA) and the I_sc was recorded before and after adding (apical side) of 10⁻⁵ M benzamil. The benzamil-sensitive component of I_sc was used as an estimate of transcellular sodium transport.

• **Measurements of extracellular ion concentrations**
Confluent monolayers (0.33 cm²) were treated as described in the text. After 16 h of incubation extracellular Na⁺, K⁺ and Ca²⁺ concentrations were determined by removing duplicate samples of 20 µl from the apical and basolateral compartment. The Na⁺ and K⁺ content of the samples were measured by flame photometry (Eppendorf FCM 6343, Hamburg, Germany). The Ca²⁺ concentration was measured using a colorimetric test kit.
Modulation of the aldosterone-induced ENaC synthesis by changing the rate of apical Na⁺ entry (Boehringer, Mannheim, Germany).

- **Immunoprecipitation of β ENaC**
  Confluent monolayers (1.13 cm²) were treated as described in the text. 2 h before the end of the incubation period, filter cups were washed 3 times for 5 min with DMEM without methionine and subsequently labeled at 37°C for 2 h by apical addition of 0.2 mCi/filter [³⁵S]Methionine (ICN Pharmaceuticals, Irvine, CA). After incubation, the cells were washed, scraped and immunoprecipitated by incubation with affinity purified β ENaC antisera as previous described in detail (5). The immunoprecipitated proteins were resuspended in 25 µL Laemmli sample buffer, and denatured 5 min at 95°C. Next, 20 µl of the samples was loaded on 7% (wt/vol) SDS-polyacrylamide gel and electrophoresed. The gel was stained for 10 min at 65°C with 0.25% (wt/vol) Coomassie Brilliant blue, 10% (vol/vol) acetic acid, 50% (vol/vol) methanol, destained two times for 10 min at 65°C with 7% (vol/vol) acetic acid, 25% (vol/vol) methanol, rinsed in water, incubated two times for 10 min with DMSO, and two times for 15 min with 20% (wt/vol) 2,5-diphenyloxazole (Sigma Chemical, St.Louis, MO, USA) in DMSO. After two times for 5 min rinsing in water, the gel was dried, and exposed to a film with an intensifying screen at -80°C. The relative amount of ³⁵S incorporation was determined with Molecular Analyst (Biorad, Hercules, Ca., USA).

- **Chemicals**
  Benzamil was obtained from Research Biochemical International (Natick, MA, USA). All other chemicals were obtained from Sigma (Sigma Chemical, St.Louis, MO, USA). Benzamil and aldosterone were dissolved in ethanol, which final concentration never exceeded 0.1% (vol/vol). Aldosterone was added to the apical and basolateral sides, whereas benzamil was added to the apical side only.

- **Statistics**
  Results are given as means ± SEM. For all experiments, statistical significance was determined by analysis of variance (ANOVA), in case of significance, individual groups
were compared by contrast analysis according to Fisher. The level of statistical significance was set at $p<0.05$.

Results

Effect of the rate of apical Na$^+$ influx on the aldosterone-stimulated benzamil-sensitive $I_{sc}$

Primary cultures of rabbit CNT and CCD cells were used to determine whether changes in the rate of apical Na$^+$ influx have an effect on transcellular Na$^+$ transport, measured as benzamil-sensitive $I_{sc}$. Stimulation of the rate of apical Na$^+$ influx was accomplished by short-circuiting the monolayers for 16 h by flooding the apical and basolateral compartments with culture medium, to establish electrical contact. As a result the apical membrane of the CCD and CNT cells will be hyperpolarized, which will stimulate the apical Na$^+$ entry. Reduction of apical Na$^+$ entry was accomplished by apical exposure of the monolayers to benzamil for 16 h. Incubation of the monolayers with aldosterone ($10^{-7}$ M, both sides) for 16 h significantly ($p<0.05$) increased the benzamil-sensitive $I_{sc}$ by $320 \pm 49\%$, whereas in chronic short-circuited monolayers no effect of aldosterone was observed ($117 \pm 14\%$ of control benzamil-sensitive $I_{sc}$, $p>0.1$) (Fig.1).

After removing the short-circuiting situation for 1 h there was no recovery of the aldosterone-stimulated benzamil-sensitive $I_{sc}$ (data not shown). Furthermore, short-circuiting the monolayers had no effect on basal benzamil-sensitive $I_{sc}$ ($72 \pm 10\%$ of control levels, $p>0.1$). When the apical Na$^+$ influx was blocked by exposure to benzamil for 16 h, the benzamil-sensitive $I_{sc}$ was doubled compared to control level. However, no significant effect was apparent on the aldosterone-induced benzamil-sensitive $I_{sc}$ ($335 \pm 74\%$ and $320 \pm 49\%$ for benzamil and aldosterone exposure and aldosterone exposure alone, respectively). In addition, the doubling of the benzamil-sensitive $I_{sc}$ by benzamil took also place under SC conditions. After 16 h of incubation, the pH of the apical compartment reduced to 5.6 in untreated monolayers, whereas during short-circuiting of the monolayers the extracellular pH remained 7.4.
Modulation of the aldosterone-induced ENaC synthesis by changing the rate of apical Na⁺ entry

Figure 1: Effect of apical Na⁺ entry on the benzamil-sensitive short-circuit current (Isc) across primary cultures of rabbit CNT and CCD cells. Unstimulated and aldosterone (Aldo) (10⁻⁷M, both sides) stimulated monolayers were for 16 h short-circuited (SC), incubated with benzamil (Benz) (10⁻⁵ M, apical side), or short-circuited and incubated with benzamil. After 16 h of exposure, each filter was washed 3 times with normal medium and Isc was measured in an Ussing chamber. Values were normalized by those obtained for the control cells (13 ± 2 μA/cm²). Data are expressed as means ± SEM of at least 6 filters; *, significant different from control (p<0.05).

To exclude that the acidification of the apical compartment is the major regulator in the stimulatory effect of aldosterone, we combined chronic short-circuiting with chronic benzamil treatment. In this latter condition, blockage of the apical Na⁺ entry at pH of 7.4, the short-circuiting induced inhibition of aldosterone-stimulated transcellular Na⁺ transport is also not realized.

Effect of extracellular ion concentrations on transepithelial Na⁺ transport

Next, we determined the Na⁺, K⁺ and Ca²⁺ concentrations in the extracellular medium of control and short-circuited monolayers, treated without or with aldosterone. Table 1 shows
that after 16 h of incubation, the apical Na\(^+\) concentration of untreated and aldosterone treated monolayers decreased from 140 mM to 82 mM and 59 mM, respectively, while apical K\(^+\) concentration increased from 5 mM to 32 mM and 38 mM, respectively. The apical Ca\(^{2+}\) concentration in unstimulated and stimulated condition is decreased from 1.0 mM to 0.36 mM and 0.47 mM, respectively. The influence of these extracellular ion concentrations on the modulation of the aldosterone-induced benzamil-sensitive \(I_{sc}\) was examined by mimicking the described circumstances. 80 mM Na\(^+\) or 30 mM K\(^+\) in the medium during short-circuiting and aldosterone treatment had no stimulating effect on benzamil-sensitive \(I_{sc}\) (50 ± 5% and 106 ± 27%, respectively).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>Aldosterone</th>
<th>Aldosterone +SC</th>
<th>Aldosterone +SC +Benzamil</th>
</tr>
</thead>
<tbody>
<tr>
<td>apical [Na(^+)]</td>
<td>81.8 ± 3.9</td>
<td>59.4 ± 3.6 *</td>
<td>149.2 ± 4.0 *</td>
<td>154.9 ± 4.0 *</td>
</tr>
<tr>
<td>apical [K(^+)]</td>
<td>32.2 ± 2.2</td>
<td>37.8 ± 5.1</td>
<td>6.3 ± 0.1 *</td>
<td>4.8 ± 0.1 *</td>
</tr>
<tr>
<td>apical [Ca(^{2+})]</td>
<td>0.36 ± 0.02</td>
<td>0.47 ± 0.04</td>
<td>1.02 ± 0.04 *</td>
<td>1.03 ± 0.03 *</td>
</tr>
</tbody>
</table>

Moreover, when the extracellular Ca\(^{2+}\) concentration was reduced by the Ca\(^{2+}\) chelator EGTA (0.8 mM) or the intracellular Ca\(^{2+}\) concentration by the Ca\(^{2+}\) chelator BAPTA-AM (10 \(\mu\)M) for 16 h, the Na\(^+\) channel activity did not change in aldosterone-stimulated short-
circuited monolayers (117 ± 25% and 54 ± 31%, respectively). Taken together, the extracellular Na⁺, K⁺ or Ca²⁺ concentrations did not influence the aldosterone induced ENaC activity of rabbit CNT and CCD cells.

Effect of the rate of apical Na⁺ influx on aldosterone-stimulated β rbENaC protein levels

The aldosterone-stimulated rbENaC proteins were measured after changing the driving force for apical Na⁺ entry. The β rbENaC protein levels were determined by radioactive immunoprecipitations of this 97 kD protein. The results of a representative immunoblot of immunoprecipitated β rbENaC from [35S]methionine labeled primary cultures of rabbit CNT and CCD are shown in figure 2.

Figure 2: Representative immunoprecipitations of β rbENaC in 16 h short-circuited (SC), benzamil (Benz) (10⁻⁵ M, apical side), or short-circuited and benzamil treated primary cultures of rabbit CCD and CNT cells in control and aldosterone (Aldo) (10⁻⁷ M, both sides) stimulated conditions. At the end of the stimulation period, cells were labeled with [35S]methionine for 2 h.

Aldosterone treatment for 16 h increased the β rbENaC protein level by 310 ± 51% (Fig.3). After 16 h short-circuiting, the β rbENaC protein level remained unaffected. The inhibitory effect of chronic short-circuiting the monolayers on the aldosterone-induced benzamil-sensitive I₄ₒ₆ as showed above, is accompanied by a significant decrease of aldosterone stimulated β rbENaC protein synthesis from 310 ± 51 % to 115 ± 12% of control levels. Furthermore, in monolayers exposed for 16 h to benzamil, the β rbENaC protein synthesis is increased to a comparable protein level (282 ± 68%) as found in aldosterone-induced
monolayers. In the combined condition of short-circuiting the monolayers, benzamil and aldosterone, the β rbENaC protein synthesis is stimulated to the same level (286 ± 45%). Thus also on protein level, benzamil treatment overcame the inhibitory effect on aldosterone stimulation by short-circuiting the monolayers.

![Graph showing protein levels of β rbENaC](image)

**Figure 3:** Effect of apical Na⁺ entry on the synthesis of β ENaC in primary cultures of rabbit CNT and CCD cells. Levels of β rbENaC were determined by immunoprecipitations of [35S]methionine labeled short-circuited (SC), benzamil (Benz) (10⁻⁵ M, apical side), or short-circuited and benzamil treated primary cultures of rabbit CNT and CCD cells in control and aldosterone (Aldo) (10⁻⁷ M, both sides) stimulated conditions. Values were normalized by those obtained for the control cells. Data are means ± SEM of at least 4 experiments; *, significant different from control (p<0.05).

**Discussion**

This study examines the influence of the rate of apical Na⁺ entry on aldosterone regulation of transepithelial Na⁺ transport. In primary cultures of rabbit kidney cortical
collecting system, aldosterone exposure stimulates an increase in benzamil-sensitive Na\(^+\) transport, resulting from an increase of \(\alpha\) and \(\beta\) ENaC protein synthesis (5). In the present study, we have demonstrated that the aldosterone-stimulated rbENaC transcription or translation process is blocked by long-term short-circuiting the monolayers. It is likely that short-circuiting the monolayers increases the intracellular Na\(^+\) concentration due to an increase in the apical Na\(^+\) influx by hyperpolarization of the apical membrane (6, 9, 17). Our study further shows that the rate of apical Na\(^+\) entry, has a significant effect on ENaC expression, since benzamil treatment overrules the inhibitory effect of short-circuiting on aldosterone stimulated Na\(^+\) transport. This feedback inhibition is mediated either by a decrease in \(\beta\) rbENaC synthesis or by an increase in \(\beta\) rbENaC degradation.

The mechanisms by which intracellular Na\(^+\) concentration might affect ENaC activity are unclear. Chronic short-circuiting the monolayers, and thereby increased intracellular Na\(^+\) concentration, may inhibit the aldosterone-induced ENaC expression via regulatory elements on the \(\alpha\), \(\beta\) and/or \(\gamma\) ENaC gene(s), for example via a yet unidentified Na\(^+\) responsive element. Many regulatory pathways, controlled by [Na\(^+\)]\(_i\), are potentially involved in the feedback regulation of ENaC. In salivary duct cells, Komwatana et al. (20, 21) have identified a \(G_0\) protein as the mediator of a Na\(^+\) feedback system. This described model for feedback regulation is as follows: cytosolic Na\(^+\) binds to an intracellular Na\(^+\) receptor, activating the \(G_0\) protein and the \(\alpha\) subunit of the \(G_0\) protein causes the ubiquitine-protein ligase, Nedd4, to ubiquinate and inactivate ENaC (16). In *Xenopus laevis* oocytes, Na\(^+\) feedback inhibition is present together with Nedd4 dependent regulation of ENaC, but does not require G protein function (15). Nedd4 proteins contain WW domains, which can bind to the PY motifs of \(\beta\) and \(\gamma\) ENaC subunits, and ubiquitinitates the ENaC C-termini, leading to endocytosis and degradation of ENaC. This feedback inhibition model, could be applied to the present study in rabbit cortical collecting system. Short-circuiting of the monolayers will result in a higher [Na\(^+\)]\(_i\) and Na\(^+\) binds in a concentration dependent manner to an intracellular Na\(^+\) receptor, which activates Nedd4 and leads to degradation of ENaC subunits. Mutations in the PY motifs associated with Liddle syndrome, an inherited form of salt-sensitive hypertension, interfere also with the feedback regulation by intracellular Na\(^+\). (7, 13, 18).
Other feedback loops possibly involved in the downregulation of ENaC have also been studied in the past. Chalfant et al. (4) showed that in lipid bilayers rENaC currents were dependent on intracellular pH and were not influenced by changes in extracellular pH. In A6 cells (36) as well as in rat cortical collecting tubules (27), changes in intracellular pH correlate positively to changes in Na⁺ current and transepithelial conductance. An important finding in our study was that an extracellular pH between 7.4 and 5.6 is not a dominant factor in aldosterone stimulated ENaC synthesis, since benzamil overcomes the inhibitory effect of short-circuiting on aldosterone-stimulated ENaC expression. In the short-circuiting situation, aldosterone-induced ENaC expression can also be decreased under control of reduced ATP levels. The rise in intracellular Na⁺ will increase the energy used by the Na/K pump, leading to a decrease in ATP levels. Downregulation of ENaC as a result of increased demand for ATP by Na/K pump is described by Frindt et al. (10). In addition, during benzamil exposure a rise in ATP can be involved in the stimulatory effect on ENaC expression. Another proposed factor involved in feedback inhibition is intracellular Ca²⁺. In the primary cultures of CNT and CCD there is no effect on benzamil-sensitive Isc after buffering of the intracellular or extracellular Ca²⁺ concentrations of the short-circuited and aldosterone exposed monolayers. The literature on experiments with increased cytoplasmic Ca²⁺ concentrations reports several discrepancies. In rat kidney, there is no direct effect of Ca²⁺ measured in inside-out patches, whereas in studies with vesicles of toad bladder and in rabbit cortical collecting tubules an increase in Ca²⁺ leads to a reduced amiloride-sensitive Na⁺ influx (9, 12).

A conclusion of our data is that an increase in cell volume, induced by increased Na⁺ influx, prevents the cell to react properly to aldosterone. Apparently, cell volume control has a higher priority then responding to aldosterone. Recently, also the role of the cell volume-sensitive kinase sgk (serine-threonine kinase) in ENaC regulation is described (22, 26, 29). Sgk is rapidly and strongly upregulated by aldosterone in rat cortical collecting duct. In addition coexpression of sgk with ENaC in Xenopus laevis oocytes stimulated ENaC activity approximately 7 fold. The sgk transcription level correlates negatively with cell volume (35). Cell swelling associated degradation of sgk also provides a possible explanation for the inhibition of aldosterone induced Na⁺ transport, which we observed in short-circuited cells.
Blocking of Na⁺ entry by benzamil may lead to cell shrinkage, and this could stimulate the accumulation of sgk and thereby ENaC activity. It is of interest to note, that modulation of Na⁺ influx by short-circuiting A6 cells in the absence of aldosterone has an opposite effect as we report here for rabbit primary cultures of CNT and CCD cells in the presence of aldosterone. Rokaw et al. (31) showed that decreasing Na⁺ influx decreases Iₑₑ and increasing Na⁺ influx increases Iₑₑ. These observations were made in the absence of aldosterone. Therefore, intracellular Na⁺ concentration may have dual effects depending on the absence or presence of aldosterone.

In conclusion, in the present study we have demonstrated that in primary cultures of the rabbit cortical collecting system the aldosterone transcription/translation process can be inhibited by chronic short-circuiting the monolayers. Chronic benzamil can overcome this inhibitory effect. The obvious explanation is feedback inhibition by an increase of the intracellular Na⁺ concentration as a result of hyperpolarization of the apical membrane. Further experiments are needed to delineate the molecular mechanism behind this feedback inhibition by intracellular Na⁺.

References

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Modulation of the aldosterone-induced ENaC synthesis by changing the rate of apical $Na^+$ entry

CHAPTER 4

Intracellular sodium concentrations at various transcellular Na\(^+\) transport rates in rabbit cortical collecting system.

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Submitted
Summary

Na⁺ reabsorption in the renal cortical collecting system is a two step process consisting of apical entry via the benzamil-sensitive ENaC channel and basolateral exit via the ouabainsensitive Na⁺,K⁺-ATPase. Both transporters must function in a concerted manner in order to maintain low intracellular Na⁺ levels ([Na⁺]ᵢ) during transcellular Na⁺ transport. In this study, [Na⁺]ᵢ was measured with the fluorescent probe SBFI during variation in the Na⁺ transport rate in rabbit connecting and cortical collecting tubules cultured on permeable filters. Inhibition of apical Na⁺ entry by benzamil decreased the [Na⁺]ᵢ significantly from 8.0 ± 0.1 to 4.8 ± 0.3 mM. Similar results were obtained by removal of Na⁺ from the apical and basolateral compartments. Inhibition of ion transport by ouabain increased [Na⁺]ᵢ to 38 ± 4 mM, which could be partially blocked by luminal benzamil. Basolateral perfusion with Na⁺ free medium suggests that the Na⁺/Ca²⁺ exchanger contributes significantly to the observed changes in [Na⁺]ᵢ. Aldosterone stimulated transepithelial Na⁺ transport by 263 ± 44% compared to control level, and this increase in Na⁺ transport rate was accompanied with a higher steady state in [Na⁺]ᵢ (9.0 ± 0.6 and 13.2 ± 0.6 mM for control and aldosterone stimulated cells, respectively). In conclusion, the data presented here suggest that regulation of influx via ENaC as well as efflux via Na⁺,K⁺-ATPase is realized with only small variations in [Na⁺]ᵢ.

Introduction

The connecting tubular and cortical collecting ductal cells (CNT and CCD) play a key role in the final part of Na⁺ reabsorption of salt in the kidney. Na⁺ (re)absorption across epithelial cells of the distal part of excretory systems, such as distal nephron, distal colon and ducts of exocrine glands, is a two stage process [11; 15]. Na⁺ entry across the apical membrane of the cell via the amiloride-sensitive epithelial sodium channel (ENaC) is followed by Na⁺ extrusion across the basolateral membrane via the Na⁺,K⁺-ATPase. This Na⁺ pump maintains a high intracellular K⁺ concentration ([K⁺]ᵢ) and a low intracellular Na⁺ concentration ([Na⁺]ᵢ), which provides a favorable electrochemical gradient for Na⁺-coupled transport processes. Although Na⁺ entry is a downhill process, this passive diffusion via
ENaC is rate limiting, saturable and highly selective for sodium [13; 8]. Na⁺,K⁺-ATPase provides the driving force for Na⁺ transport and is coupled to hydrolysis of ATP, which provides the energy for three Na⁺ ions transported out of the cell and two K⁺ ions taken up by the cell [2].

The steroid hormone aldosterone plays a major role in the regulation of the transepithelial Na⁺ transport, and thus plays a key role in the regulation of Na⁺ balance, extracellular volume and blood pressure. In rabbit CNT and CCD cells, the aldosterone-stimulated sodium transport is characterized by an early response within 3 hours, and a late response lasting 6 to 16 hours [4]. In addition to this aldosterone regulation, we have recently demonstrated that intracellular Na⁺ concentration is thought to be involved in feedback regulation of ENaC [5]. Stimulation of the apical Na⁺ entry, by long-term short-circuiting the monolayers, suppressed the aldosterone-stimulated Na⁺ transport while in the presence of benzamil this inhibitory effect was not observed. Therefore, it is of interest to examine the effect of aldosterone on [Na⁺].

The aim of the present study was to identify the pathways of Na⁺ influx and in primary cultured CNT and CCD cells. We measured the [Na⁺], under various transport conditions induced by Na⁺ transport blockers, benzamil and ouabain as well as by replacing extracellular Na⁺ iso-osmotically with N-methyl-D-glucamine-Cl (NMDG).

Materials and Methods

- **Chemicals**
Collagenase A and hyaluronidase were obtained from Boehringer (Mannheim, Germany). SBFI/AM and pluronic F127 were from Molecular Probes (Eugene, OR). Benzamil was obtained from Research Biochemical International (Natick, MA, USA). All other chemicals and hormones were from Sigma (St. Louis, MO).

- **Solutions**
NaCl-buffer consisted of (mM): 140 NaCl, 2 KCl, 1 K₂HPO₄, 1 KH₂PO₄, 1 MgCl₂, 1 CaCl₂, 5 glucose, 5 L-alanine, 10 HEPES/Tris (pH 7.4). Krebs-Henseleit Buffer (KHB) contained
(mM): 128 NaCl, 5 KCl, 1.2 MgSO₄, 1 CaCl₂, 2 NaH₂PO₄, 10 glucose, 10 Na-acetate, 4 L-lactate, 1 L-alanine and 20 HEPES/Tris (pH 7.4). Na⁺-free buffer was obtained by isosmotic replacement of NaCl in NaCl-buffer with N-methyl-D-glucamine-Cl (NMDG). Benzamil, aldosterone and amphotericin B were dissolved in ethanol. Final solvent concentrations never exceeded 0.1% (v/v).

- **Primary cultures of rabbit kidney cortical collecting system cells**
Rabbit kidney connecting tubule and cortical collecting duct cells, hereafter referred to as cortical collecting system cells, were isolated from New Zealand white rabbits (~ 0.5 kg body weight) by immunodissection with monoclonal antibody R2G9 and set in primary culture on either transparent (fluorescence measurements) or non-transparent (Ussing chamber experiments), permeable filters (0.33 cm²; Costar, Cambridge, MA), as previously described in detail [1]. Cell monolayers reached confluency at day 3 and were used between 5 and 8 days after seeding. Confluence of the monolayer was routinely checked by determining the transepithelial potential difference and resistance with two sterile chopstick-like electrodes connected to a Millicell-ERS meter (Millipore, Etten-Leur, Netherlands).

- **Fluorescence measurements**
Rabbit cortical collecting system cells, grown to confluency on permeable and transparent filters (0.4 μM pore size), were loaded with SFBI in DMEM/F-12 medium containing 10 μM SBFI/AM, 0.02% (w/v) pluronic F127, 3 mM probenecid and 5% (v/v) decomplemented fetal calf serum for 60 min at 37°C. Subsequently, filters were washed three times with NaCl-buffer containing 3 mM probenecid, mounted in a thermostated (37°C) perfusion chamber and placed on the stage of an inverted microscope (Diaphot, Nikon, Tokyo, Japan). A gravity controlled superfusion system maintained perfusion speeds of 120 and 200 ml/h of the apical and basolateral compartments. Fluorescence measurements were performed with a long working distance objective (Fluor 60x; N.A. = 0.7; Nikon). Dynamic video imaging was carried out with the MagiCal hardware and the TARDIS software provided by Joyce Loebl (Tyne & Wear, UK), as described previously [9]. Cells were excited intermittently at 340
and 380 nm and SBFI fluorescence emission was monitored at 492 nm. After correction for background the ratio 340 and 380 frames were obtained. The interframe interval between the ratio frames was 6.4 s. On every filter, 8-16 single cells were monitored simultaneously and analyzed individually.

\[ [\text{Na}^+]_i = \frac{K_d \times Rbf \times (R-R_{\text{min}})}{(R_{\text{max}}-R)} \]

[Na\textsuperscript{+}]i was calculated at the level of single cells according to the formula derived by Harootunian et al. [7]: [Na\textsuperscript{+}]i = Kd * Rbf * [(R-R_{\text{min}})/(R_{\text{max}}-R)]. Following each experiment calibration constants were obtained by addition of 5 μM amphoterin B to the apical compartment containing [Na\textsuperscript{+}] between 0 and 140 mM, respectively. A Kd of 18 mM was assumed [10] and the value of Rbf, which is equivalent to the ratio of the 380 nm excitation fluorescence emission intensities measured in the absence and presence of 140 mM Na\textsuperscript{+} in the extracellular medium after the addition of 5 μM amphoterin B, was 1.0.

- **Transcellular Na\textsuperscript{+} transport measurements**

Rabbit cortical collecting system cells, grown to confluency on permeable filters (0.4 μM pore size), were incubated at 37°C for 16 h in the absence or presence of aldosterone (10\textsuperscript{-7} M, basolateral side). Subsequently, filter cups were mounted between two half-chambers and bathed at 37°C with NaCl-buffer. The solutions bathing the monolayer were connected via agar bridges and Ag-AgCl electrodes to a voltage-clamp current amplifier (Physiological Instruments, San Diego, CA) and the short-circuit current (Isc) was recorded in the absence and presence of 10\textsuperscript{-5} M benzamil (apical side). The benzamil-sensitive component of Isc was used as an estimate of transcellular Na\textsuperscript{+} transport.

- **Statistical analysis**

In all experiments data were obtained from at least three different isolations of cortical collecting system cells and are given as means ± SEM for the number of filters (transcellular Na\textsuperscript{+} transport) or the number of single cells ([Na\textsuperscript{+}], experiments) measured. Statistical differences between the mean values were determined by analysis of variance (ANOVA), and in the case of significance individual groups were compared by contrast analysis according to Fisher. Results were considered significant when P < 0.05.
Chapter 4

Results

Intracellular sodium concentrations ([Na⁺]ᵢ) were estimated with the fluorescent dye SBFI using transparent filters on which rabbit cortical collecting system cells were grown to confluency. The SBFI fluorescence 340/380 nm ratio values were obtained from single cells by dynamic fluorescence microscopy and converted to [Na⁺]ᵢ values, as described in the Materials and Methods section.

Calibration of Na⁺ probe SBFI

The fluorescence signal of the SBFI-loaded monolayers decreased rapidly in time, indicating that the dye leaked out of the cell and/or that the fluorescence signal was rapidly bleached. However, in the presence of 3 mM probenecid, an inhibitor of organic anion transport, the rate of decrease in the fluorescence signal was reduced to an acceptable level (data not shown). Probenecid was, therefore, continuously present during the loading procedure and subsequent experiments. Confocal microscopy revealed that the dye was evenly distributed in the cytosol and occasionally distinct fluorescent spots were observed within the cell, suggesting that SBFI is only partly compartmentalized (Fig. 1).

Figure 1: Confocal image of SBFI-loaded rabbit cortical collecting cells grown to confluency on transparent permeable supports. Monolayers, loaded with the fluorescent Na⁺ indicator SBFI, were superfused apically and basolaterally with NaCl medium at 37°C. The fluorescence emission was captured at 492 nm after excitation at 364 nm. The image shown is from a representative experiment. Bar = 10 μm.
The fluorescence ratio values obtained from single cells were calibrated by increasing the Na⁺ permeability of the cell with 5 μM amphotericin B and subsequently [Na⁺] was increased from 0 to 140 mM in both compartments to saturate intracellular SBFI, as shown in figure 2A for a representative experiment.

Figure 2: Calibration of fluorescent ratio’s of SBFI-loaded rabbit cortical collecting cells grown to confluency on transparent permeable supports. Monolayers, loaded with the fluorescent Na⁺ indicator SBFI, were placed in a gravity controlled and thermostated (37°C) perfusion chamber and were superfused apically and basolaterally. The temporal dynamics of the fluorescence signals were analyzed simultaneously in 8-16 individual cells by digital-imaging microscopy. The fluorescence emission intensities at 492 nm, after intermittent excitation at 340 and 380 nm, were ratioed as a measure of Na⁺-sensitive fluorescence. At the indicated point, 5 μM amphotericin B was added to the apical and basolateral compartment. Subsequently, the extracellular Na⁺ concentration was stepwise increased from 0 to 140 mM and the resultant fluorescence ratio was calculated (left). In the right panel, the fluorescence ratio is plotted as a function of [Na⁺]₀. From this curve an apparent affinity constant of SBFI for sodium was calculated of 17.1 ± 1.7 mM.
In the absence of extracellular Na\(^+\) a minimal fluorescence ratio of 0.53 ± 0.01 (N=41) was obtained and subsequent stepwise elevation of Na\(^+\) increased the ratio in a dose-dependent manner to a maximal value of 1.15 ± 0.01 (N=41). An apparent affinity constant of SBFI for Na\(^+\) was calculated of 17 ± 2 mM. As depicted in figure 2B, in the cortical collecting system cells SBFI can be used to monitor [Na\(^+\)], reliably between 1 and 40 mM.

**Inhibition of Na\(^+\) transport**

In the present study, we examined whether variations in Na\(^+\) fluxes leads to changes in [Na\(^+\)]. Therefore, two different inhibitors of Na\(^+\) transport were used. First, benzamil, inhibitor of ENaC-mediated apical Na\(^+\) entry, and second, ouabain, inhibitor of Na\(^+\),K\(^+\)-ATPase-mediated basolateral Na\(^+\) efflux, as shown in figure 3.

![Figure 3: The effect of benzamil and ouabain on [Na\(^+\)], of rabbit cortical collecting system cells grown to confluency on transparent permeable supports. For [Na\(^+\)], measurements monolayers were loaded with the fluorescent Na\(^+\) indicator SBFI, as described in the legend of figure 2. The monolayers were perfused with NaCl medium and at the indicated point 10 μM benzamil (A) or 1 mM ouabain (B) was added to the apical or basolateral compartment respectively. Data shown are from representative experiments and the accompanying [Na\(^+\)], values are as indicated.](image)
Addition of benzamil reduced, whereas ouabain increased the fluorescence ratio of SBFI. The corresponding [Na⁺] was calculated from 340/380 fluorescence ratio using the calibration curve, and the mean values calculated from at least three different isolations are presented in figure 4.

![Graph showing the effect of benzamil and extracellular sodium on [Na⁺] of rabbit cortical collecting system cells treated with or without ouabain.](image)

**Figure 4:** The effect of benzamil and extracellular sodium on [Na⁺] of rabbit cortical collecting system cells treated with or without ouabain. For [Na⁺] measurements, monolayers were loaded with the fluorescent Na⁺ indicator SBFI, as described in the legend of figure 2. The monolayers, perfused with NaCl medium (A) or with basolateral medium containing 1 mM ouabain (B), were exposed to benzamil (10 μM, apical) or to the medium in which Na⁺ was iso-osmotically replaced by N-methyl-D-glucamine in the apical compartment (apical NMDG) or basolateral compartment (basolateral NMDG). Data are mean ± SEM of at least 21 cells. * Significantly different from control (P<0.05). ** Significantly different from benzamil (P<0.05).

In the absence of these inhibitors, the cultured cortical collecting system cells exhibited a [Na⁺] of 8.0 ± 0.1 mM (N=50). Inhibition of apical Na⁺ entry by benzamil (10 μM, apical side) significantly decreased the basal [Na⁺], to 4.8 ± 0.3 mM (N=47, P < 0.05). On the other hand, inhibition of basolateral Na⁺ extrusion by ouabain (1 mM, basolateral side) significantly increased the basal [Na⁺], to 38 ± 4 mM (N=21). Like in benzamil exposed cells, a fall in [Na⁺], was obtained by replacing apical Na⁺ iso-osmotically with N-methyl-D-glucamine (NMDG). Replacement of basolateral Na⁺ reduced [Na⁺], to the same extent.
Besides ouabain treatment of the monolayers under control condition, we also investigated the effect of ouabain on [Na\(^+\)], in the presence of benzamil or NMDG. As shown in figure 4B, benzamil significantly attenuated the ouabain-induced increase of [Na\(^+\)], to 26 ± 3 mM (N=40, P<0.05). Interestingly, in the continued presence of NMDG apical or basolateral, addition of ouabain resulted in a significant lower [Na\(^+\)], than in monolayers treated with ouabain in the presence of benzamil.

**Stimulation of transcellular Na\(^+\) transport**

The primary cultures responded to aldosterone (10\(^{-7}\) M, basolateral side) with an increase in benzamil-sensitive I\(_{sc}\), as a measure for transcellular Na\(^+\) transport, as shown in figure 5A. After 16 h, the stimulatory response to aldosterone was 263 ± 44% of control (N=8, P < 0.05). These maximally elevated Na\(^+\) transport rates were accompanied by a 47% increase in [Na\(^+\)], (9.0 ± 0.6 and 13.2 ± 0.6 mM for control and aldosterone-treated cells, respectively, N=36, P < 0.05) (Fig.5B).

![Graph showing the effect of aldosterone on Isc and [Na\(^+\)]i](image)

*Figure 5: The effect of aldosterone on Isc and [Na\(^+\)]i of rabbit cortical collecting system cells grown to confluency on transparent permeable supports. For short-circuit current (I\(_{sc}\)) measurements monolayers were placed in an Ussing chamber, as described in the Materials and Methods. For [Na\(^+\)]i measurements monolayers were loaded with the fluorescent Na\(^+\) indicator SBFI, as described in the legend of figure 2. The monolayers were incubated in the absence or presence of 10\(^{-7}\) M aldosterone for 16 h, and subsequently I\(_{sc}\) and [Na\(^+\)]i were estimated. Data shown are mean ± SEM of at least 8 filters and 36 cells respectively. * Significantly different from control (P<0.05).*
Intracellular sodium concentrations at various transcellular Na⁺ transport rates

Discussion

Primary cultures of rabbit cortical collecting system were used to measure [Na⁺]ᵢ under various active NaCl transport rates. The use of SBFI and the ability to alter excitation wavelengths rapidly from 340 to 380 nm enabled us to monitor [Na⁺]ᵢ during changes in Na⁺ transport rates. In the present study, we report a basal steady state [Na⁺]ᵢ of 8.0 mM. This value is comparable to previous reported values of 7 mM [16], 8.6 mM [6], in rabbit urinary bladder and lower than the 12 mM reported for rabbit colon [14]. The difference lies in the techniques used since previous values were all collected using ion-selective microelectrodes.

We demonstrated here that inhibition of the apical Na⁺ influx by the ENaC blocker benzamil resulted in a decrease in [Na⁺]ᵢ. Moreover, a comparable reduction in [Na⁺]ᵢ was obtained by replacing apical Na⁺ with NMDG. This last finding suggests that apical influx of Na⁺ occurs primarily via the benzamil-sensitive Na⁺ channels in the cortical collecting system. In addition, also reducing basolateral Na⁺ by replacement with NMDG led to a decrease in [Na⁺]ᵢ, indicative for a significant Na⁺ influx via the basolateral membrane.

In this study, it is further shown that inhibition of the basolateral Na⁺ efflux by the Na⁺,K⁺-ATPase blocker ouabain raises [Na⁺]ᵢ, as expected. Moreover, this increase could only partially (+41%) be blocked by benzamil, indicating that the ouabain-induced increase in [Na⁺]ᵢ, is also due to a basolateral Na⁺ entry mechanism. Moreover, ouabain-induced increase in [Na⁺]ᵢ can be blocked to basal levels by replacement of basolateral Na⁺ with NMDG. It is likely that this basolateral Na⁺ entry system is the basolateral located Na⁺/Ca²⁺ exchanger, since in previous studies has been shown that this exchanger is highly expressed in CNT cells and functions as an efflux pathway in active transcellular Ca²⁺ transport. In contrast to untreated cells, in ouabain treated cells the observed effect of replacing either apical or basolateral Na⁺ with NMDG is significantly different from the benzamil-induced decrease in [Na⁺]ᵢ. It is possible that non-selective cation transporters are activated by cell swelling due to the increase in [Na⁺]ᵢ by ouabain.

In the present study we clearly demonstrate that in rabbit CNT and CCD [Na⁺]ᵢ increased to 13 mM as a result of aldosterone stimulation of the transcellular Na⁺ transport rate. In a study in rat CCD, a [Na⁺]ᵢ value of 22 mM was found after putting the rats on low
Na⁺ diet [12], but they did not measure the [Na⁺], in unstimulated tubules. In rabbit urinary bladder the 10 fold increase in short-circuit current by aldosterone resulted in only a doubling of [Na⁺], to about 20 mM [6].

In CCD from aldosterone-repleted adrenalectomized rabbits it was demonstrated by [³H]ouabain binding that in the range of 15 mM [Na⁺], to 140 mM [Na⁺], the number of Na⁺,K⁺-ATPase units in the basolateral membrane increased linearly with [Na⁺]. This increase was independent of RNA or protein synthesis [3], which suggests the presence of a latent intracellular pool of Na⁺ pumps. In addition, in CCD from adrenalectomized rabbits without aldosterone repletion the specific ouabain binding was reduced and did not further increase with rising [Na⁺]. In primary cultures of rabbit CNT/CCD we demonstrated that in response to aldosterone the increase in [Na⁺], to 13 mM, which is 147% compared to control levels, is accompanied by a 264% increase in ENaC activity. In a previous study we reported that an increase in the rate of apical Na⁺ entry inhibited aldosterone-induced increase in ENaC expression by a feedback inhibition mechanism [5]. The results described here suggest that this feedback inhibition operates within a narrow range of changes in [Na⁺]. Therefore, [Na⁺], plays a role in regulating the apical Na⁺ entry via ENaC and in insertion of addition Na⁺ pumps in the basolateral membrane. This dual role of [Na⁺], can be considered as a strong cell volume regulatory mechanism, which is needed to prevent the cell from Na⁺ overload and cell swelling which can lead to cell death.

References


CHAPTER 5

The epithelial sodium channel (ENaC) is intracellularly located as a tetramer

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Summary

The epithelial sodium channel (ENaC) plays an important role in Na⁺ homeostasis by determining the Na⁺ transport rate in so-called end-organs as the renal collecting duct, distal colon, salivary and sweat gland ducts. ENaC is formed by heteromultimerization of three homologous subunits, termed α, β and γ ENaC. The number of subunits and stoichiometry remains a matter of debate. In this study, sucrose gradient analysis of Xenopus laevis oocytes expressing rENaC revealed that ENaC forms heterotetramers, when the membrane fraction was solubilized in 0.1% (wt/vol) Na-deoxycholate. However, solubilization of the membrane proteins in higher concentrations of detergents dissociated the ENaC subunits of the tetramers in dimers. Co-immunoprecipitation studies with FLAG-tagged ENaC subunits suggest that during dissociation of ENaC tetramers the composition of dimers is completely random. Glycosylation studies show that the ENaC subunits are retarded in the ER and pre-Golgi, whereas only a small fraction is inserted into the plasma membrane. Immunocytochemical analysis confirmed that ENaC is primarily located intracellularly. In addition, these findings are not restricted to the oocyte expression system, since identical results were found in rabbit connecting tubule and cortical collecting duct cells in primary culture and in rabbit colon.

Introduction

The epithelial sodium channel (ENaC) plays a major role in the transepithelial (re)absorption of sodium in collecting ducts, distal colon, salivary and sweat ducts, respiratory tract and taste buds [1]. ENaC belongs to the DEGenerin/Epithelial Na⁺ Channel (DEG/ENaC) superfamily [2], which is involved in a broad spectrum of cellular functions such as electrolyte transport, mechanosensation and neurotransmission. Members of this family are found in nematodes, flies, snails and several vertebrates, including mammals. The heteromultimeric ENaC is formed by three structurally related subunits α, β and γ ENaC [3]. Like all members in the DEG/ENaC superfamily, each subunit has a large extracellular loop, two short membrane-spanning domains and short cytoplasmic N and C termini [4]. The
The epithelial sodium channel (ENaC) is intracellularly located as a tetramer. Conserved proline-rich motifs in the C terminal region of the α ENaC subunit are essential for apical localization, whereas the proline-rich motifs in the C terminal region of the β and γ ENaC subunits are essential for endocytosis of the channel [5, 6]. In the *Xenopus laevis* oocyte expression system the α ENaC subunit is sufficient to induce channel activity, whereas coexpression of α ENaC with β and γ ENaC subunits are necessary to obtain maximal channel current [3]. The stoichiometry of the hetero-oligomeric ENaC complex is still a matter of debate. Biochemical and biophysical studies suggested two different subunit compositions: a tetrameric structure consisting of two α, one β and one γ subunits [7, 8] and an eight to nine hetero-oligomer [9, 10, 11], respectively.

The aim of the present study was to determine, independently, the subunit stoichiometry of the ENaC complex when expressed in *Xenopus laevis* oocytes. To this end, membranes of oocytes overexpressing α, β and γ rENaC were solubilized and subjected to sucrose gradient centrifugation. The results show that in oocyte membranes, rENaC is primarily present as a tetrameric structure. In this study, we demonstrate in *Xenopus laevis* oocytes, by using immunoblotting of isolated total and plasma membranes, glycosylation studies and immunohistochemistry, that only a small fraction of rENaC subunits is expressed in the plasma membrane. Furthermore, this finding is not restricted to the oocyte expression system since analysis of rabbit kidney and rabbit colon also hint at intracellular location.

Materials and Methods

- **Expression constructs and transcription**

The cRNAs encoding rENaC subunits and FLAG-tagged rENaC subunits (kindly provided by D. Firsov and B.C. Rossier) were linearized with Not I (α, γ), Bgl II (β, αFLAG, βFLAG) or PVU II (γFLAG) and *in vitro* transcribed using T7 RNA polymerase (Gibco, Paisley, UK) (α, γ) or SP6 RNA polymerase (Promega, Madison, WI) (β, αFLAG, βFLAG, γFLAG) according to Promega's Protocols and Principles guide (1991), except for that the nucleotide triphosphates and 7-methyl-diguanosine triphosphate were used at a final concentration of 1 mM. The cRNAs were purified and dissolved in diethylpyrocarbonate.
(DEPC)-treated MilliQ water. The integrity of the cRNAs was checked by agarose gel electrophoresis and the concentrations were determined spectrophotometrically.

- **Isolation and injection of Xenopus laevis oocytes**
  Oocytes were isolated from *Xenopus laevis* and defolliculated by digestion at room temperature for 2 h with 2 mg/ml collagenase A (Boehringer Mannheim, Germany). Stage V and VI oocytes were selected and stored at 18°C in modified Barth's solution (MBS) [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 10 mM HEPES-KOH (pH 7.5), 0.8 mM MgSO4, 0.3 mM Ca(NO3)2, 0.4 mM CaCl2, supplemented with 50 μg/ml gentamycin]. Oocytes were coinjected with 3 ng α, β and γENaC subunit cRNA in a final volume of 50 nl and stored at 18°C in MBS. Two days after injection the oocytes were analyzed.

- **Membrane isolation of Xenopus laevis oocytes**
  Sixty injected or non-injected oocytes were homogenized in 1 ml of homogenization buffer (HbA) [20 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 5 mM NaH2PO4, 1 mM EDTA, 80 mM sucrose, 1mM phenylmethylfluoride (PMSF), 5 μg/ml leupeptin and pepstatin] and centrifuged at 200 g for 5 min at 4°C to remove yolk proteins. Next, a total membrane preparation was isolated by centrifugation at 14000 g for 30 min at 4°C.

- **Membrane isolation of rabbit colon**
  Colonic cells obtained from young New Zealand white rabbits (0.5 kg body weight) were scraped in 10 ml PBS and spun down for 5 min at 200 g at 4°C. The cells were homogenized in 5 ml of HbA using a pestle and centrifuged at 1000 g for 10 min 4°C to remove nuclei and cell debris. Subsequently, the supernatant was centrifuged at 200,000 g for 30 min at 4°C.

- **Immunoprecipitation of β ENaC from primary cultures of rabbit CNT/CCD cells**
  Primary cultures of connecting tubule and cortical collecting duct cells cells were immunodissected from kidney cortex of young New Zealand white rabbits with Mab R2G9 [15]. Cells were grown to confluence for 5-8 days on permeable supports, and then used for immunoprecipitation of the β ENaC protein, as described previously [15].
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- **Glycosylation studies**
  Endo H or PNGase F treatment of total membranes of oocytes, rabbit colon or samples of immunoprecipitated β ENaC from rabbit CNT and CCD, were performed according to the protocol described by manufacture (New England Biolabs, Beverly, MA).

- **Plasma membrane isolation**
  Vitelline membranes of 30 oocytes were removed and oocytes were coated with 1%(wt/vol) colloidal silica, Ludox Cl (Sigma Aldrich, St. Louis, MO) in freshly made MES buffered salt solution (MBSS) [20 mM MES, 80 mM NaCl-NaOH (pH 6.0)] washed twice with MBSS and incubated in 0.1% (vol/vol) poly acrylic acid (Sigma Aldrich) [12]. During both incubations, oocytes were rotated slowly for 30 min at 4°C. After two times washing with MBS, the oocytes were homogenized in HbA. Then the plasma membranes were isolated using 4 washing steps with slow centrifugation (3x 13.5 g, 1x 24 g, 1x 38 g) at 4°C for 30 sec, after each step 1 ml of the top of the sample was removed and 1 ml of HbA was added. After the last centrifugation step, HbA was removed and plasma membranes were spun down at 14000 g for 20 min at 4°C.

- **Sucrose gradient centrifugation**
  Isolated total membranes were dissolved in 300 µl solubilization buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% (vol/vol) glycerol, 1 mM PMSF, 5 µg/ml leupeptin and pepstatin] containing 0.1% or 1% (wt/vol) Na-deoxycholate or 1% (wt/vol) digitonin, as indicated, for 1 h at 37°C and centrifuged at 14000 g for 1h at 4°C to remove undissolved membranes essentially according to Neely and Agre [13]. Sedimentation by gradient centrifugation was done essentially as described by Jung et al. [14]. Linear 10-35% (wt/vol), 15-45% (wt/vol) and 15-55% (wt/vol) sucrose density gradients were prepared from 0 and 55% (wt/vol) sucrose stock solutions in 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.1% (vol/vol) Triton X-100, 1 mM PMSF, 5 µg/ml leupeptin and pepstatin. Samples of membrane proteins in Na-deoxycholate or digitonin were layered on top of the gradients and centrifuged at 150,000 g in a swing out rotor (SW 60) for 16 h at 7°C. Fractions of 200 µl were then carefully collected from top to bottom, concentrated by 10% (wt/vol) TCA.
precipitation at 4°C and analysed by immunoblotting. As protein markers a mixture of alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), catalase (232 kDa) and apoferritin (443 kDa) (Sigma, St.Louis, Mo.) was used and sedimented using the same protocol.

- **Immunoprecipitation**
  Equivalents of 30 μl pre-washed Protein G-agarose beads (Pharmacia, Upsala, Sweden) were pre-incubated O/N at 4°C with 2 μl of monoclonal FLAG antibody (m2; Sigma) in IPP500 [500 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.1% (wt/vol) NP-40, 0.1% (vol/vol) tween-20, 1 mM PMSF, 5 μg/ml leupeptin and pepstatin] and 0.1% (vol/vol) BSA. Membrane proteins of 30 non-injected or injected oocytes with either αFLAG, β and γ rENaC cRNA or α, βFLAG and γ rENaC cRNA or α, β and γFLAG rENaC cRNA in 1% deoxycholate (150 μl) were incubated with washed antibody-bound protein G-agarose beads in 850 μl IPP100 [500 mM NaCl, 10 mM Tris (pH 8.0), 0.1% NP-40, 0.1% tween-20, 1 mM PMSF, 5 μg/ml leupeptin and pepstatin] O/N at 4°C, washed three times with IPP 100 and dissolved in Laemmli [2% (wt/vol) SDS, 50 mM Tris (pH 6.8), 12% (vol/vol) glycerol, 0.01% (wt/vol) Coomassie Brilliant Blue, 100 mM dithiothreitol (DTT)]. Samples were loaded on a 7% (wt/vol) SDS-polyacrylamide gel and electrophoresed, blotted and subjected to immunodetection with β ENaC antibody [15].

- **Immunoblotting**
  Protein samples were denaturated in Laemmli buffer for 30 min at 37°C and loaded on a 7% (wt/vol) SDS-polyacrylamide gel, electrophoresed and transferred to PVDF membranes (Millipore Corporation, Bedford, MA) by standard procedures. Marker proteins from sedimentation gradients were analyzed by a 9% (wt/vol) SDS-polyacrylamide gel and Coomassie Brilliant Blue staining. Blots were incubated with 1:1000 diluted affinity-purified β ENaC antibodies or 1:500 diluted affinity-purified α or γ ENaC antibodies [15] and subsequently incubated in 1:10000 dilution of purified rabbit-anti-guinea pig IgG conjugated to horse radish peroxidase (Sigma) supplemented with 1% (wt/vol) non-fat dried milk. Proteins were visualized using enhanced chemiluminescence (Pierce, Rockford, IL). The intensity of the protein bands was measured using Molecular Analyst (Biorad, Hercules, Ca).
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- **Immunohistochemistry**
At two days after injection, oocytes were stripped from remaining vitelline membranes. These oocytes, colon and kidney obtained from New Zealand white rabbits were incubated for 2 h in 1% (wt/vol) periodate lysine paraformaldehyde fixative (PLP), dehydrated and embedded in paraffin. Sections of 5µm were cut by cryostat, dewaxed, rehydrated and incubated in preboiled citrate buffer (pH 6.0), containing 0.01 M citric acid and 0.01 M Na-citrate, cooling down to room temperature for 30 min. After three washes with TN (0.1 M Tris-HCl (pH 7.5) and 0.15 M NaCl), the sections were blocked with TNB (TN and 0.5% (vol/vol) blocking reagent (NEN life science products, Boston, MA)) for 30 min and incubated O/N at 4°C with a 1:100 dilution of affinity-purified polyclonal β ENaC [15] in TNB. The sections were washed three times for 5 min in TNT, and subsequently incubated for 1 h at RT with a 1:300 dilution of goat anti-guinea pig Alexa (Molecular Probes, Eugene, OR) in TNT. The sections were again washed three times, then dehydrated in 50-100% (vol/vol) of methanol and mounted in mowiol 4-88, containing 2.5% (wt/vol) NaN₃. Photographs were taken with a Zeiss Axioskop microscope equipped with epifluorescent illumination on Kodak EPH P1600X films.

**Results**

Subunit stoichiometry of ENaC expressed in oocytes
To study ENaC subunit assembly, total membranes of oocytes co-injected with α, β and γ rENaC cRNA were isolated, solubilized using different detergent concentrations and finally subjected to sucrose gradient sedimentation centrifugation. The localization of ENaC subunits in the different fractions was revealed by immunoblotting (Fig.1A). The sedimentation marker proteins alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), catalase (232 kDa) and apoferritin (443 kDa), which were loaded on parallel sucrose gradients, were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (Fig.1C). The sedimentation profile in figure 1B shows that the peak intensities for α, β and γ rENaC after 1% Na-deoxycholate solubilization were present in fraction 8-9 using a 10-35% sucrose gradient, which corresponds to the distribution of the 200 kDa standard.
Figure 1: Oligomerization state of rENaC using 1% Na-deoxycholate. Membranes from α, β and γ rENaC expressing oocytes were solubilized in 1% Na-deoxycholate, and sedimented on 10-35% sucrose gradient. A, fractions were collected and immunoblotted for α, β and γ ENaC. B, corresponding quantification of the rENaC subunits. C, sedimentation pattern of the standard protein markers subjected to a parallel sucrose gradient.

Since each ENaC subunit, expressed in oocytes, is present in an 89-97 kDa form, our results suggest a dimeric form of the protein when 1% Na-deoxycholate was used as detergent. After solubilization of membranes in 0.1% Na-deoxycholate, α, β and γ rENaC peaked in
The epithelial sodium channel (ENaC) is intracellularly located as a tetramer fraction 9-10 using a 15-45% sucrose gradient, reflecting more the distribution of the 443 kDa standard (Fig.2).

Figure 2: Oligomerization state of rENaC using 0.1% Na-deoxycholate. Membranes from α, β and γ rENaC expressing oocytes were solubilized in 0.1% Na-deoxycholate, and sedimentated on 15-45% sucrose gradient. A, fractions were collected and immunoblotted for α, β and γ ENaC. B, corresponding quantification of the rENaC subunits. C, sedimentation pattern of the standard protein markers subjected to a parallel sucrose gradient.

Also when ENaC was solubilized in 1% digitonin, the peak intensity for rENaC was found in fraction 9 (Fig.3). These results indicate that in total membranes of ENaC expressing oocytes, the rENaC complex is composed of at least four subunits. For a better separation of large complexes, we also performed a 25-55% sucrose gradient centrifugation, after 1% digitonin solubilization (Fig.4). Interestingly, the ENaC complex formed no higher order oligomers than the four subunit oligomerization.
Figure 3: Oligomerization state of rENaC using 1% digitonin. Membranes from α, β and γ rENaC expressing oocytes were solubilized in 1% digitonin, and sedimented on 15-45% sucrose gradient. A, fractions were collected and immunoblotted for β ENaC. B, corresponding quantification of the β rENaC subunit.

Figure 4: Sucrose gradient analysis of rENaC using 25-55% sucrose gradient. Membranes from α, β and γ rENaC expressing oocytes were solubilized in 1% digitonin, and sedimented on 25-55% sucrose gradient. A, fractions were collected and immunoblotted for β ENaC. B, corresponding quantification of the β rENaC subunit. C, sedimentation pattern of the standard protein markers subjected to a parallel sucrose gradient.
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Subunit assembly of ENaC

To investigate the subunit assembly of ENaC in more detail, the rENaC subunits were tagged with a FLAG epitope. Membranes of oocytes co-injected with FLAG-tagged α, β and γ rENaC were solubilized in 0.1% Na-deoxycholate and subjected to 10-35% sucrose gradient sedimentation centrifugation. Immunoblot with β rENaC antibody of the fractions taken from this gradient revealed that FLAG-tagged rENaC peaked in fraction 11 corresponding with a tetramer, like untagged rENaC (Fig. 5).

Figure 5: Oligomerization state of FLAG-tagged rENaC using 0.1% Na-deoxycholate. Membranes from FLAG-tagged α, β and γ rENaC expressing oocytes were solubilized in 0.1% Na-deoxycholate and sedimented on 15-45% sucrose gradient. A, fractions were collected and immunoblotted for β ENaC. B, corresponding quantification of the β rENaC subunit.

Since dimeric structures of ENaC are present after 1% Na-deoxycholate solubilization, it is interesting to know whether there is a preference in subunit binding in these dimers. Therefore, membranes of oocytes co-expressing α, β and γ rENaC, of which only the α, β or γ ENaC subunit is FLAG-tagged, were solubilized in 1% Na-deoxycholate and immunoprecipitated with monoclonal FLAG antibodies. Subsequently, immunoblotting for
the presence of β rENaC shows that β rENaC co-immunoprecipitated with α rENaC as well as with γ rENaC (Fig.6), albeit more β rENaC protein was co-immunoprecipitated with α rENaC than with γ rENaC.

![Figure 6: Co-immunoprecipitation of FLAG-tagged rENaC. Membranes from non-injected oocytes and from αFLAG, β and γ rENaC or α, βFLAG and γ rENaC or α, β and γFLAG rENaC expressing oocytes were solubilized in 1% Na-deoxycholate, and immunoprecipitated with Flag antibodies. The precipitates were subsequently immunoblotted with β ENaC antibody.](image)

**Localization of ENaC**

To determine the extent of expressed rENaC subunits in the plasma membrane, plasma membranes of oocytes expressing rENaC were isolated with an improved method in which high yields of relatively pure plasma membranes are obtained by low-speed sedimentation after coating with silica beads [12]. Immunoblotting of total membranes and plasma membranes revealed that only a minor fraction of the rENaC subunits is present in the plasma membrane (Fig.7).

![Figure 7: Immunoblot analysis of ENaC expressed in total and plasma membranes. Total and plasma membranes from oocytes expressing rENaC were isolated. Subsequently, equivalents of 5, 2 or 1 oocyte(s) were immunoblotted with β ENaC antibody.](image)
The epithelial sodium channel (ENaC) is intracellularly located as a tetramer.

Subsequently, to determine the degree of maturation of ENaC and the cellular compartment in which the subunits are localized, we treated membrane fractions with the glycanases Endoglycosidase H (Endo H) and N-glycosidase F (PNGase F). Endo H cleaves only high mannose-glycosylated forms and some hybrid oligosaccharides from N-linked glycoproteins. Endo H sensitivity is, therefore, an indication that proteins remain in the ER and pre-Golgi, and are not further processed. On the other hand, PNGase F cleaves nearly all types of N-glycans, and is therefore used as control. Figure 8A shows that β ENaC subunits are completely digested by both Endo H and PNGase F, indicating that almost all the β ENaC subunits are retained in the ER or pre-Golgi compartments. In addition, we examined whether Endo H and PNGase F sensitivity is restricted to ENaC expressed in oocytes.

**Figure 8:** Digestion of ENaC by glycanases. β ENaC treated with and without PNGase F or Endo H from oocytes expressing α, β and γ rENaC (A), primary cultures of CNT and CCD (B), and rabbit colon (C). A and C were analyzed by immunoblotting with β ENaC antibody. B, primary cultures of CNT/CCD were pulse-labeled with [35S]methionine for 2h and subsequently immunoprecipitated with β ENaC antibody.

In figure 8B and figure 8C it is shown that deglycosylation of β ENaC subunits from rabbit CNT and CCD and colon is equally effective for Endo H and PNGase F treatment. Therefore, we also tested the ENaC localization by immunohistochemistry (Fig.9). In ENaC expressing oocytes, rabbit kidney cortex and colon immunopositive staining was mainly present in cytoplasmic domains, confirming the results obtained with the glycanases.
Chapter 5

Discussion

In the present study, we obtained evidence that the ENaC complex, upon expression of α, β and γ rENaC Xenopus laevis oocytes sediments after solubilization as a complex of four subunits. An important factor in the study of native multimeric structure of ENaC is the detergent concentration with which the membrane proteins are extracted from the lipid bilayer and kept in solution. After solubilization with two different detergents, 0.1% Na-deoxycholate and 1% digitonin, we observed a sedimentation pattern that resembled the pattern of a 443 kDa standard protein. Independent of the percentage sucrose in the gradient, ENaC sedimented in one peak, corresponding to a tetrameric complex. However, solubilization in 1% Na-deoxycholate disrupted the interaction between subunits in the tetrameric structure, as indicated by a dissociation in dimers. In view of the reported preferential ENaC subunit assembly in tetramers with a 2α-1β-1γ stoichiometry [7,8], and the possibility to form a functional channel by co-expression of α together with β or γ ENaC.

Figure 9: Immunohistochemical localization of β ENaC. Distribution of β ENaC in oocyte expressing α, β and γ rENaC (A), rabbit kidney cortex (B) and rabbit colon (C). A: strong β ENaC staining of cytoplasm in the oocyte expressing ENaC is shown. B: β ENaC staining was found mainly at intracellular sites in the CNT as well as in the CCD. C: in colon, β ENaC staining distributed throughout the villus tip.
The epithelial sodium channel (ENaC) is intracellularly located as a tetramer [16]. We examined whether the tetrameric complex dissociates into αβ and αγ dimeric complexes. Using 1% Na-deoxycholate as detergent, our results showed that β ENaC co-immunoprecipitated with α ENaC as well as with γ ENaC. Thus, we proposed that during ENaC dissociation there is a random combination between the subunits in the dimeric form.

The tetrameric subunit stoichiometry observed in the present study is in agreement with the studies of Firsov et al. [7] and Kosari et al. [8], in which biophysical methods and expression of concatamers were used. On the other hand, an eight to nine subunit stoichiometry was suggested by Cheng et al. [9], Eskandari et al. [10], and Snijder et al. [11] using both, biophysical and biochemical assays. Cheng and co-workers [9] examined the oligomerization state of hENaC in COS-7 cells by using 1% digitonin and sucrose gradient analysis. After pulse labeling of 30 min and chase of 4 h in COS-7 cells hENaC mainly assembled as a heteromultimer of about 950 kDa, whereas in the beginning of the chase hENaC oligomerized as dimers or trimers. Previously it has been demonstrated that ENaC has a short turnover time, with half-lives of about 1 h for at least two of the subunits [17, 18]. This suggested that the ~950 kDa ENaC complex, as found at the end of the 4 h chase period, has an increased channel stability. For these reasons, it is likely that ENaC is associated with proteins involved in the turnover of ENaC, which may contribute to the determined size of the ENaC complex. In addition, it would also be possible that oligomerization is dependent on the cellular localization of ENaC. This could mean that ENaC is only forming a stable nine subunit stoichiometry when it has reached the plasma membrane.

Since in the present sucrose gradient analysis α, β and γ rENaC subunits were analyzed in a total membrane fraction, we also examined ENaC composition in the plasma membrane. However, the majority of ENaC protein was present intracellularly, whereas only a small amount could be detected on the plasma membrane. It was, therefore, technically not possible to determine by sucrose gradient centrifugation the oligomerization state of ENaC in the plasma membrane. In addition, β ENaC subunits were completely deglycosylated by Endo H, indicative of retardation in the ER or pre-Golgi. These findings could be confirmed by immunohistochemistry and identical results were obtained with rabbit CNT/CCD and colonic cells. In these mammalian cells β ENaC was also found in a core-glycosylated Endo

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H-sensitive form. The results presented here are in agreement with the findings of Valentijn et al. [19], who demonstrated that posttranslational maturation of ENaC in the ER-Golgi is the limiting step in the synthesis of functional channels in oocytes. Moreover, observations in mice suggest that a low-Na\(^+\) diet, and thereby a higher aldosterone level, stimulates the translocalization of ENaC from intracellular pools to the apical plasma membrane [20]. In addition, also in rabbits kept on a low Na\(^+\) diet immunopositive staining of ENaC was shifted to a more apical domain [15].

The above discussed inefficient processing is not restricted to ENaC, since less than 30% of newly synthesized immature chloride channel CFTR (cystic fibrosis transmembrane conductance regulator) proceeds through more complex glycosylation pathways [21]. Maybe both, ENaC and CFTR, are inefficiently processed because of the absence of assembly factors. Maturation of CFTR precursor from ER to Golgi is accompanied by about 20-fold increase of stability. This stability likely reflects acquisition of a correct tertiary structure [21]. On the basis of the results presented here, it is likely that the unfolded or partly folded state of ENaC in the ER and pre-Golgi is not correctly released from interaction with chaperones, and are rapidly delivered to the proteasome. These huge amount of immature subunits could be the explanation for the short half-lives of the ENaC subunits. Furthermore, ER retardation is very often seen in a number of genetic diseases, where expression of mutated form of these proteins fail to reach the correct destination due to misfolding and ER retardation. CFTR and Aquaporin-2 (AQP-2) mutants, which causes Cystic Fibrosis (CF) and Nephrogene Diabetes Insipidus (NDI), respectively, are well studied examples [21, 22]. The ER serves as a quality-control organelle for proper processing and assembly of membrane proteins [23].

Moreover, examination of the oligomerization state of ENaC in the plasma membrane by freeze-fracture electron microscopy suggests that ENaC consists of eight to nine subunits [10]. The dimension of the freeze-fracture particels in ENaC expressing oocytes were used to determine that 17 ± 2 transmembrane α-helices are present in the plasma membrane forming a functional ENaC complex. Our results described in this paper suggest that ENaC assembles into tetramers in the ER or pre-Golgi. Conceptually, the plasma membrane ENaC protein complex could have resulted from an eight subunit assembly by association of two
The epithelial sodium channel (ENaC) is intracellularly located as a tetramer before they can be transported from the Golgi complex to the plasma membrane. It could, therefore, be possible that ENaC is a double-barreled channel similar to the “two subunits-two pores” stoichiometry proposed for the chloride channel CIC-0 [24].

Taken together, our results demonstrate a tetrameric structure of ENaC upon expression in oocytes. The expressed ENaC complexes are mainly located in the ER-Golgi domain, whereas only a small fraction is inserted into the plasma membrane. However, in spite of this low number of ENaC incorporation at the plasma membrane, transepithelial Na⁺ transport via ENaC can be functionally measured. It is important to characterize in detail the pathway of ENaC posttranslational processing, and in particular, the role of cellular assembly factors and chaperones in this process.

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The epithelial sodium channel (ENaC) is intracellularly located as a tetramer.


CHAPTER 6

Summarizing discussion and perspectives
Summarizing discussion and perspectives

The kidney plays an important role in the maintenance of body fluid and Na⁺ homeostasis. Every day 1.7 kg of NaCl and 180 L H₂O are reabsorbed by the renal tubules. Most of the reabsorption of Na⁺ and water (about 60-70%) takes place in the proximal tubules. However, the major sites of fine tuning the Na⁺ reabsorption are the connecting tubule and cortical collecting duct. The Na⁺ reabsorption at these sites is under hormonal control of aldosterone which acts on specific Na⁺ channels. These channels, called Epithelial Na⁺ channel (ENaC), are present in several epithelia such as frog skin, urinary bladder, sweat duct, salivary gland duct, distal colon and respiratory epithelial cells. ENaC is composed of three homologous subunits, α, β and γ and defines a new gene family, the DEG/ENaC superfamily. The initial cloning in 1994 of ENaC from rat distal colon (Canessa et al., 1994) opened new possibilities to study structural and regulatory properties of the ENaC channel.

In this thesis, we investigated the regulation of ENaC by the steroid hormone aldosterone at ENaC mRNA, ENaC protein and Na⁺ transport levels (Chapter 2). In Chapter 3 the intracellular Na⁺ concentrations were measured during changes in the Na⁺ transport rate and moreover, the effect of the rate of apical Na⁺ entry on the long-term response of aldosterone was investigated in Chapter 4. Finally, in Chapter 5, we studied the oligomeric assembly of the ENaC complex and we obtained insight in the maturation of this channel.

Primary culture of rabbit CNT/CCD as model system

The regulation of ENaC has been frequently studied in A6 cells, which are derived from amphibian kidney tissue (Kemendy et al., 1992; Rossier and Palmer, 1992; May et al., 1997). In our laboratory a primary culture of rabbit connecting tubule and cortical collecting duct cells (CNT/CCD) was developed (Bindels et al., 1991). Rabbit kidney cortex of young New Zealand white rabbits (~0.5 kg body mass) was enzymatic digested by collagenase and hyaluronidase to obtain tubules segments. The tubules were subsequently immunodissected using the monoclonal antibody R2G9, directed against a cell surface epitope specifically present on the CNT/CCD cells. Finally the immunodissected cells were cultured on
Summarizing discussion and perspectives

permeable filters to form a polarized confluent monolayer. This mammalian model system retains several characteristics of the original epithelium, including a benzamil-sensitive lumen-negative transepithelial potential difference and responsiveness to aldosterone. In conclusion, the primary culture of rabbit CNT/CCD provides an excellent system to study the effects of aldosterone on distinct levels in a highly purified cell population.

Effects of aldosterone on ENaC

In Chapter 2, we report for the first time, the time-dependent effect of aldosterone on benzamil-sensitive Na⁺ transport, ENaC mRNA and ENaC protein in a mammalian model system. Partial sequencing of rabbit α, β and γ ENaC has made it possible to design specific primers to assess the aldosterone effect on ENaC mRNA levels. Furthermore, specific polyclonal antibodies were raised against the three ENaC subunits, enabling to study the effect of aldosterone on ENaC protein levels.

Aldosterone stimulated transcellular Na⁺ transport in a time-dependent manner. After a lag time of 3 h, the first phase of aldosterone stimulation of transcellular Na⁺ transport occurs without a measurable increase in both, ENaC mRNA and protein level. This suggests that ENaC protein synthesis is not necessary for this initial effect. From 6 to 16 h there is a late response, accompanied by an increase in α, β and γ ENaC mRNA levels and an increase in α and β ENaC protein levels. Thus, the increase in γ mRNA is not translated into its protein, which agrees with previous findings in A6 cells (May et al., 1997). In contrast to the aldosterone response in A6 cells, in the primary cultures of CNT/CCD cells there is no short-term effect of aldosterone up to 2 h. This could be due to several circumstances. For instance, the differences in baseline levels of Na⁺ transport between both studies (Verrey et al., 1995) could be responsible for the time scale of the aldosterone response. Another possibility is that the short-term aldosterone effect on Na⁺ transport is absent from mammalian epithelia.

In many studies the effect of aldosterone has been reported on the abundance of the mRNA levels of α, β and γ ENaC in whole distal colon, lung, kidney or kidney derived cell lines (Champigny et al., 1994; Renard et al., 1995; Lingueglia et al., 1994; Stokes and Sigmund, 1998). These results showed that the three subunits are differently affected by
aldosterone and that the response of each ENaC subunit can be different in the various aldosterone-responsive tissues. Since we demonstrated that the aldosterone effect on the abundance of ENaC mRNA is not directly proportional to the abundance of ENaC protein, it is unknown whether the described effects of aldosterone on ENaC mRNA fully account for aldosterone-induced protein levels and channel activity. For maximal channel activity all the three ENaC subunits are required in *Xenopus laevis* oocytes (Canessa et al., 1994), it is therefore unclear why, in either primary cultures of rabbit CCT/CND cells or A6 cells, α and β ENaC protein levels are upregulated by aldosterone whereas γ ENaC protein levels remain constant. One explanation could be that the hetero-oligomeric subunit composition will be changed under control of aldosterone. However, since we showed in Chapter 5, that ENaC is mainly intracellularly located and that only a small fraction is inserted into the plasma membrane, it is also of interest to know the relative importance of increasing the α and β ENaC protein levels for ENaC activity. In recent years, several aldosterone-induced proteins (AIP) were identified, which are involved in activating or processing ENaC (Spindler et al., 1997; Spindler and Verrey, 1999; Chen et al., 1999). Instead of ENaC protein levels, it could well be that these AIP’s are the limiting factors in ENaC assembly and/or insertion of ENaC into the plasma membrane. Aldosterone stimulation of Na⁺ transport activity is a complex process and may involve the activation and/or translocation of pre-existing channels and the enhanced transcription/translation process of the three ENaC subunits.

**Transcellular Na⁺ transport and intracellular Na⁺ concentrations**

In Chapter 4, the intracellular Na⁺ concentrations ([Na⁺]) were measured, using the fluorescent probe SBFI, at various transcellular Na⁺ transport rates in the primary culture of rabbit cortical collecting system. The results show that the regulation of Na⁺ influx via ENaC as well as Na⁺ efflux via Na⁺, K⁺-ATPase occurs within small variations in [Na⁺]. In that situation influx and efflux must be well balanced, which is needed to prevent cellular Na⁺ overload and cell swelling. Another interesting finding in this study was that replacement of basolateral Na⁺ with NMDG results in reduction of [Na⁺]. This indicates that there is a significant Na⁺ influx via the basolateral membrane. Since previous studies have showed that the Na⁺/Ca²⁺ exchanger is highly expressed in the cortical collecting system where it is
involved in active Ca\textsuperscript{2+} transport (Bindels et al., 1992; Reilly et al., 1993), it is likely that this basolateral Na\textsuperscript{+} influx system is the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger.

Modulation of aldosterone-induced ENaC synthesis

In Chapter 3 of this thesis, the effect was investigated of the rate of apical Na\textsuperscript{+} entry on controlling ENaC activity and aldosterone-responsiveness. Primary cultures of rabbit CNT/CCD were short-circuited by flooding the monolayers with culture medium to stimulate apical Na\textsuperscript{+} influx or treated overnight with benzamil to block apical Na\textsuperscript{+} influx. In this system, we have demonstrated that the aldosterone-stimulated transepithelial Na\textsuperscript{+} transport and ENaC transcription/translation processes can be inhibited by prolonged short-circuiting of the monolayers. Furthermore, this inhibitory effect could be overcome by long-term benzamil treatment. Moreover, long-term benzamil treatment of control monolayers stimulated transepacellular Na\textsuperscript{+} transport and the β ENaC protein level. The obvious explanation for the effects of modulating the apical Na\textsuperscript{+} influx is feedback by [Na\textsuperscript{+}]. In Chapter 4, measurements of [Na\textsuperscript{+}], revealed that after blocking the apical Na\textsuperscript{+} influx by benzamil, [Na\textsuperscript{+}], decreases significantly from 8.0 to 4.8 mM. It is plausible that the increase in ENaC activity and β ENaC protein level by long-term benzamil treatment is due to a positive feedback mechanism mediated by this decrease in [Na\textsuperscript{+}]. On the other hand, blocking of the aldosterone-induced ENaC activity by simultaneous short-circuiting the monolayers is due to negative feedback inhibition. After long-term aldosterone stimulation of transepithelial Na\textsuperscript{+} transport, [Na\textsuperscript{+}], increased to 13 mM. However, at short-circuiting conditions, it is technically difficult to measure the [Na\textsuperscript{+}]. Nevertheless, our interpretation is that in the short-circuiting condition, [Na\textsuperscript{+}], is higher then the [Na\textsuperscript{+}], level after 16 h aldosterone stimulation and, that this high [Na\textsuperscript{+}], level prevents the onset of the aldosterone effects. This means that an increase in Na\textsuperscript{+} influx, which leads to increased [Na\textsuperscript{+}], and cell swelling, prevents the long-term effect of aldosterone.

Feedback inhibition of ENaC

After excluding the influence of acidification and extracellular Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} ion concentrations on the modulation of the aldosterone-induced ENaC activity, we suggested
that the feedback mechanisms are mediated by [Na+]. The mechanisms by which [Na+]
might affect ENaC activity is at present unclear. Suppression of aldosterone-induced ENaC
activity, accompanied by a decrease in β ENaC synthesis or an increase in β ENaC
degradation may occur via yet unidentified Na+ responsive elements in the genes coding for
ENaC subunits. A feedback inhibition model, possibly involved in ENaC regulation, is
described for salivary duct cells and Xenopus laevis oocytes (Komwatana et al., 1998;
Hybnera et al., 1999). In these feedback inhibition models, intracellular Na+ binds in a
concentration dependent manner to an intracellular Na+ receptor, which activates, possibly
via mediator G, protein, Ned4 and leads to degradation of ENaC subunits. Another
possible feedback inhibition mechanism occurs via cell volume dependent sgk
(serine/threonine kinase) expression. Sgk is rapidly and strongly upregulated by aldosterone
(Naray-Fejes-Toth et al., 1999) and coexpression of sgk with ENaC in Xenopus laevis
oocytes revealed a 7 fold stimulation of ENaC activity on the plasma membrane (Shigaev et
al., 2000; Chen et al., 1999). Furthermore sgk expression levels correlate negatively with cell
volume (Waldegger et al., 1997). Stimulation of apical Na+ entry by short-circuiting the
monolayers may lead to cell swelling and degradation of sgk and consequently in decreased
aldosterone-induced ENaC activity. On the other hand, cell shrinkage as a result of blocking
apical Na+ entry by benzamil, could stimulate the accumulation of sgk and thereby ENaC
activity. We can conclude that an increase in cell volume prevents the cell to react properly
to aldosterone and that cell volume control has a higher priority than responding to
aldosterone.

Oligomerization state of ENaC

In Chapter 5 of this thesis, Xenopus laevis oocytes were used as an expression system to
study the oligomerization state of ENaC. This system has many advantages, such as the
simplicity of isolation, maintance of these cells, and the high translation capacity. In the
Xenopus laevis oocyte expression system the α ENaC subunit is sufficient to induce channel
activity, whereas coexpression of α ENaC with β and γ ENaC subunits are necessary to
obtain maximal Na+ currents (Canessa et al., 1994). In Chapter 5, the number of subunits and
stoichiometry of the ENaC complex were investigated by using sucrose gradient analysis.
When the membrane fractions of oocytes expressing ENaC were solubilized in a low concentration detergent (0.1% (wt/vol) Na-deoxycholate), ENaC is present in a heterotetrameric form. However, by increasing the detergent concentration (to 1% Na-deoxycholate) the complex falls apart into dimers. In addition, co-immunoprecipitation studies suggest that during dissociation of ENaC tetramers into dimers the composition of the subunits is random. Since in the same study it was observed that ENaC complexes remain mostly as immature proteins in ER or early-Golgi compartments, we conclude that ENaC was intracellularly located as a tetramer. A tetrameric structure of the ENaC complex is also suggested by Firsov et al. (1998) and Kosari et al. (1998), but in their studies a multiple four subunit stoichiometry can not be excluded. An eight to nine subunit stoichiometry is reported by Cheng et al. (1998), Eskandari et al. (1999) and Snijder et al. (1998). After pulse labeling of 30 min and chase of 4 h in Cos-7 cells, Cheng and co-workers found that the human ENaC complex assembled as a heteromultimer of about 950 kDa. Each ENaC subunit was present in an 89-97 kDa form, with a short half-life of about 1 h for at least 2 of its subunits (Staub et al., 1997). This could mean that the ENaC complex is associated with proteins involved in the stability of ENaC, which may contribute to the determined size. Alternatively, ENaC is only forming a nine subunit stoichiometry when it has reached the plasma membrane. Examination of the subunit stoichiometry of ENaC in the plasma membrane by freeze-fracture electron microscopy revealed that ENaC consist of eight to nine subunits (Eskandari et al., 1999). Because of the small amount of ENaC present in the plasma membrane, determination of their oligomerization state by sucrose gradient centrifugation, like we showed for the total membrane preparation, was technically not possible. We hypothesize that the ENaC complex when inserted in the plasma membrane could have resulted from an eight subunit assembly by association of two tetramers before they can be transported out of the ER to the plasma membrane. It could, therefore, be possible that ENaC is a double barreled channel similar to the “two subunits-two pores“ stoichiometry proposed for the chloride channel CIC-0 (Jentch et al., 1999). In this model, the channel has two identical pores, that can gate independently of each other.
Chapter 6

Processing of ENaC

The results of the plasma membrane isolation and glycosylation studies in Chapter 5, showed that in *Xenopus laevis* oocytes ENaC is very inefficiently processed. The majority of ENaC protein was present intracellularly and only a small amount could be detected in the plasma membrane. These findings could be confirmed by immunohistochemistry and identical results were obtained in rabbit kidney cortex and colonic epithelium. Comparing the immunopositive staining of β ENaC in rabbits kept on a normal (Chapter 5) or a low-Na⁺ diet (Chapter 2), demonstrated that ENaC was shifted to a more apical domain in rabbits fed a low-Na⁺ diet that is associated with a higher aldosterone level. Our data agree with the immunohistochemical observations in mice, where the intracellular distribution of ENaC is stimulated by the dietary Na⁺ intake (Loffing et al., 2000).

Valentijn and co-workers (1998) demonstrated in *Xenopus laevis* oocytes that posttranslational maturation of ENaC in the ER-Golgi complex is the limiting factor in the expression of functional ENaC channels in the plasma membrane. Our findings in rabbit primary cultures of CNT/CCD, in rabbit colonic cells and *Xenopus laevis* oocytes expressing ENaC are in agreement with the findings by Valentijn et al. (1998). The α, β and γ ENaC subunits with 6, 12, and 4-5 glycosylation sites in multiple asparagine residues, respectively, were completely deglycosylated by Endo H treatment. Endo H sensitivity indicate retardation in the ER or pre-Golgi, whereas Endo F, which was used as a positive control, cleaves all types of N-glycans. Moreover, overexpression of the ENaC subunits does not overcome the blockade of the channels in the ER and does not result in an increasing number of channels in the plasma membrane. Coexpression of the three subunits, increased the half-life of the subunits in the ER, but this assembly is not enough for translocation and insertion of the ENaC complex into the plasma membrane (Valentijn et al., 1998). The rapid turnover of ENaC is sensitive to inhibitors of the proteasome and the lysosomal/endosomal degradation system (Staub et al., 1997).

The inefficient processing of transport proteins is not restricted to ENaC. For example, more than 70% of the newly synthesized immature CFTR (cystic fibrosis transmembrane conductance regulator) molecules never reach the plasma membrane. It is possible that these proteins are inefficiently processed because of the absence of limiting factors involved in
assembly or processing. However the maturation of CFTR has never been examined in native tissue or in primary cell cultures (Kopito et al., 1999). In general, abnormal levels of channels in the plasma membrane have been linked to several genetic disorders. Cystic Fibrosis (CF) and Nephrogenic Diabetes Insipidus (NDI) are well-studied examples of disorders whereby mutations in the CFTR and AQP-2 (Aquaporin-2) proteins, respectively, resulted in ER retardation and decreased stability (Kopito et al., 1999; Deen et al., 2000).

Conclusions and future perspectives

This thesis has generated new facts about the regulation of aldosterone-stimulated transcellular Na⁺ reabsorption in the distal nephron and the regulation and oligomeric assembly of the Epithelial Na⁺ Channel (ENaC) in particular. We suggest that the majority of ENaC subunits are assembled as heterotetramer in the ER and are not efficiently routed to the apical membrane. We postulate that ENaC complexes in the plasma membrane forming a “two tetramers-two pores” stoichiometry. To examine this hypothesis of an eight subunit stoichiometry, it will be necessary to increase the expression levels of ENaC at the plasma membrane by, for instance, coexpression with sgk. A sufficient number of ENaC complexes in the plasma membrane are required to perform sucrose gradient analysis with pure dissolved plasma membranes. Alternatively, the oligomerization state of ENaC can be investigated by sucrose gradient analysis of radioactive labeled proteins. Finally, for high resolution structural analysis of ENaC two dimensional crystals are needed to visualize the structure of the channel.

There are many questions that remain to be addressed. The emphasis will be on unraveling the mechanism(s) involving targeting of ENaC and the role of aldosterone in these processes. Long-term regulation of aldosterone-induced transcellular Na⁺ transport in rabbit CNT/CCD, is accompanied by an increase in α and β ENaC protein levels. However, since there is no intracellular shortage of channels, we question the importance of the increased ENaC protein levels for ENaC activity. Therefore, it will be interesting to characterize in detail the pathway of ENaC posttranslational processing, and in particular, the role of cellular assembly factors and chaperones in this process. Moreover, it is
important to identify unknown cDNA's corresponding to aldosterone-induced or suppressed mRNA's and elucidate the cellular events related to the induction of these and previously characterized factors to the activation of ENaC.

We conclude that the apical Na\(^+\) influx and basolateral Na\(^+\) efflux are tightly regulated to maintain [Na\(^+\)], within a narrow range. Furthermore, the rate of Na\(^+\) influx modulates the effect of aldosterone on transepithelial Na\(^+\) transport and ENaC expression. The aldosterone-stimulated Na\(^+\) transport can be blocked by long-term short-circuiting the monolayers, likely due to an increased [Na\(^+\)]. Further experiments are needed to delineate the molecular mechanism(s) behind this feedback regulation of ENaC by [Na\(^+\)]. Identification of the factors involved this feedback mechanism will provide new insights into how aldosterone stimulates transepithelial Na\(^+\) transport.

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Abbreviations

AIP’s aldosterone-induced proteins
ANOVA analysis of variance
ATL thin ascending limb of Henle’s loop
ATP adenosine 5’-triphosphate
Bapta-AM 1,2-bis(2-aminophenoxy)ethane-N’,N’,N’,N’,-tetraacetic acid
bp basepairs
BSA bovine serum albumin
CCD cortical collecting duct
CNT connecting tubule
DCT distal convoluted tubule
DEPC diethylpyrocarbonate
DMEM Dulbecco’s modified Eagle’s medium
DMSO dimethylsulfoxide
DTL thin descending limb of Henle’s loop
EDTA ethylenediaminetetraacetate
ENaC epithelial sodium channel
ER endoplasmic reticulum
FCS fetal calf serum
FITC fluorescence isothiocyanate
HbA homogenization buffer A
HEPES N-2-hydroxyethyl-piperazine-N’-2-ethansulfonic acid
IMCD inner medullary collecting duct
Isc short-circuit current
[K+]i intracellular potassium concentration
kDa kilodalton
KHB Krebs-Henseleit buffer
MBS modified Barths solutions

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MBSS  MES buffered saline for silica
Mab  monoclonal antibody
[Na+],  intracellular sodium concentration
Nedd4  neuronal cells expressed developmentally downregulated
NMDG  N-methyl-D-glucamine-Cl
OMCD  outer medullary collecting duct
PBS  phosphate buffered saline
PCT  proximal convoluted tubule
PLP  paraformaldehyde-lysine-periodate
PMSF  penylmethylsulfonyl fluoride
PST  proximal straight tubule
RT-PCR  reverse transcriptase-polymerase chain reaction
SBFI  sodium-binding benzofuran isophthalate
SDS  sodium dodecyl sulphate
sgk  serum and glucocorticoid-regulated kinase
TAL  thick ascending limb of Henle’s loop
TBS  tris buffered saline
TBST  tris buffered saline with tween-20
Tris  tris(hydroxy-methyl)aminomethane
Publications


Samenvatting in het Nederlands voor niet-vakgenoten


Om de regulatie van het natriumtransport en van ENaC in het bijzonder te kunnen bestuderen hebben we als modelsysteem gebruik gemaakt van gekweekte verbindingen- en verzamelbuiscellen van het konijn. De cellen afkomstig uit de konijnneieren worden van elkaar losgemaakt en met behulp van een cel-specifiek antiglomerulus worden de verbindingen- en verzamelbuiscellen gescheiden van de andere celtypen. Deze verbindingen- en verzamelbuiscellen worden vervolgens uitgezaaid op filters en vormen na enkele dagen een enkele gepolariseerde cellaag, vergelijkbaar met de oorspronkelijke verbindingen- en
verzamelbuiscellen met een urine- en bloedkant. Over deze gekweekte ceellaag kan het natrium transport gemeten worden.

Het epitheliale Natrium Kanaal (ENaC) is een eiwit dat in 1994 ontdekt is. Door deze ontdekking kan de regulatie van het natriumtransport nu ook op het niveau van het eiwit zelf en van de boodschapper van het eiwit bestudeerd worden. Bovendien kan nu ook bekeken worden waar het eiwit zich in de cel bevindt. Hierbij wordt onderscheid gemaakt tussen de drie bouwstenen waaruit ENaC bestaat, α-, β-, en γ-ENaC. In dit proefschrift is de regulatie door aldosteron bestudeerd van het natriumtransport en van ENaC in het bijzonder. Aldosteron speelt een belangrijke rol in de regulatie van het bloedvolume en dus de bloeddruk. Het blijkt uit ons onderzoek en uit dat van anderen dat de drie ENaC-bouwstenen verschillend door aldosteron gereguleerd worden en bovendien dat de regulatie sterk afhangt van het weefsel waarin het bestudeerd wordt. Tijdens de bestudering van de samenstelling van het ENaC-complex zijn we er achter gekomen dat het kanaal, vermoedelijk in een nog niet complete vorm, zich voornamelijk in de cel bevindt. Slechts een klein gedeelte van totale hoeveelheid ENaC komt terecht in de plasmamembraan, alwaar het zijn functie kan uitoefenen. Hierdoor kunnen we ons afvragen hoe belangrijk de toename van de hoeveelheid eiwit en boodschapper van het eiwit door aldosteron is als er al zoveel van in de cel aanwezig is. Het lijkt erop dat het veel belangrijker is dat door aldosteron het complete ENaC-complex gevormd kan worden en uiteindelijk in de plasmamembraan geplaatst kan worden om het natrium te kunnen reabsorberen.

Een andere interessante vinding in dit proefschrift is het feit dat het aldosteron-gestimuleerde natrium transport geremd kan worden door gelijktijdig met de aldosteronbehandeling de natrium-instroom te bevorderen. Doordat waarschijnlijk de concentratie van het natrium in de cel stijgt, vindt er negatieve terugkoppeling plaats op het functioneren van het kanaal. De stimulatie van ENaC wordt geremd en de concentratie van het natrium in de cel kan dan niet verder stijgen. Dit voorkomt dat de cel te veel Na⁺ en water opneemt en daardoor zou zwellen en zelfs zou kunnen barsten. Andersom, een afname van de natrium-instroom door het gebruik van een specifieke remmer het ENaC volledig te blokkeren, gaf aanleiding tot een toename van het aantal ENaC-kanalen.
Verder is in dit proefschrift de concentratie van het natrium in de cel bestudeerd onder verschillende Na⁺-transportsnelheden. We kunnen uit deze studie concluderen dat de natrium-concentratie in de cel tussen nauwe grenzen constant wordt gehouden doordat de natrium-instroom aan de urine kant via ENaC en de natrium-uitstroom aan de bloed kant via de Na⁺, K⁺-pomp aan elkaar gelijk zijn. We hebben echter ook gevonden dat er een tweede natrium-instroomsysteem moet zijn aan de bloedzijde van de cel.

Belangrijke onderzoeksvragen die overblijven zijn welke eiwitten er door aldosteron aangemaakt of gestimuleerd worden. Recent zijn er enkele eiwitten gevonden die betrokken zijn bij de stimulatie van ENaC door aldosteron. Deze en nieuwe eiwitten moeten onderzocht worden op hun betrokkenheid in het aldosteron-gestimuleerde natrium-transport. Gedacht wordt aan eiwitten die belangrijk zijn om het kanaal in de plasmamembraan te krijgen, door te helpen bij de vorming van de juiste samenstelling van het ENaC-complex en bij het transport van ENaC naar de plasmamembraan. Hierbij is het belangrijk te weten in welke vorm het ENaC in de plasmamembraan het natrium transporteert.
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Curriculum Vitae