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Aldosterone and Thyroid Hormone Modulation of α1-, β1-mRNA, and Na,K-Pump Sites in Rabbit Distal Colon Epithelium. Evidence for a Novel Mechanism of Escape from the Effect of Hyperaldosteronemia

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Summary. Aldosterone and thyroid hormone regulation of Na,K-pump biosynthesis has been examined in the distal colon epithelium of rabbits. Qualitative analysis of α-subunit isoform distribution (α1, α2, α3) detected only the α1-mRNA in the distal colon epithelium and outer renal medulla, while all three isoforms were observed in rabbit brain. A low-sodium diet led to a rise in serum aldosterone from 0.6 nM to 1.4–1.9 nM and an increase of α1-mRNA to 162%, β1-mRNA to 120%, and the number of Na,K-pump units as determined by specific [^{3}H]-ouabain binding to 182% of control by the second day of the diet. While aldosterone levels remained elevated, a spontaneous decrease in serum levels of T_3 and T_4 to 50–60% of control from the third day of the diet was followed by downregulation of β1-mRNA to 55–67%, α1-mRNA to 63–105%, and of [^{3}H]-ouabain binding to 103% of control, suggesting that a reduced rate of synthesis of the β1-subunit is rate limiting for Na,K-pump biosynthesis. Substitution with T_3 (10 μg/kg) at the seventh day with transient restoration of serum T_3 to control levels, led to rapid accumulation of β1-mRNA to 152%, of α1-mRNA to 135%, and of the number of Na,K-pump units to 153% of control. This is consistent with thyroid hormone having a permissive role for the aldosterone stimulation of Na,K-pump biosynthesis. Reduced rates of β-subunit transcription due to low thyroid hormone levels appear to provide a mechanism for escape from the effect of hyperaldosteronemia on the number of Na,K-pump units.

Key Words sodium pump · Na,K-ATPase · aldosterone · thyroid hormone · distal colon epithelium

Introduction

In mammals, mineralocorticoids augment the rate of active sodium reabsorption across tight epithelia bordering the extracellular space such as the distal colon epithelium [5, 7, 8, 12, 14, 29, 33, 39] or cortical collecting duct in the kidney [1, 4, 32, 34, 36, 42]. The rate-limiting steps for transcellular sodium transport across these epithelia are the passive flux of Na^+ ions into the cytoplasm through amiloride-sensitive Na^+ channels in the luminal membrane and the active transport of Na^+ from the cytoplasm to blood which is driven by the Na,K-pump in basolateral membranes [21, 34]. Provided the supply of ATP is sufficient, the Na,K-pump is capable of matching the active flux of Na^+ across the basolateral membrane to the apical sodium conductance. In the early or acute aldosterone-induced response, the turnover rate of existing Na,K-pumps in the basolateral membrane is increased in response to upregulation of the luminal membrane sodium conductance through activation of pre-existing latent Na^+ channels [9, 34]. Chronic adaptation of the amount of Na,K-pump protein [20, 21] and the area of basolateral membrane per cell [42] is a slow process developing over several days in response to a sustained increase in aldosterone secretion and in transepithelial Na^+ flux across the epithelium [32].

The effect of aldosterone on the biosynthesis of Na,K-ATPase has been studied in isolated toad bladder [11] or cultured epithelial cells [19, 24, 40, 41] and recently in rat cardiocytes [16]. In amphibian epithelia, aldosterone (100–300 nM) modulates the level of expression of Na,K-ATPase as measured by changes in mRNA of the α-subunit and β-subunit and in protein abundance [11, 19, 41]. In rat cardiocytes α1- and β1-mRNA were induced with an EC_{50} of 1–2 nM aldosterone [16]. There is evidence for direct interaction of the aldosterone-receptor complex with the gene to enhance transcription rates of the Na,K-pump proteins in cultured A6 cells [40]. However, it is uncertain whether the cytoplasmic Na^+ activity acts as a mediator for the action of aldosterone since a correlation between intracellular Na^+ and the amount of Na,K-ATPase has been es-

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tablished in rat kidney [20] and other tissues [32, 34]. In mammalian kidney, triiodothyronine (T_3) enhances the sensitivity of Na,K-ATPase to aldosterone in adrenalectomized rabbits and rats [1, 4], but in amphibian cells there seems to be antagonism between thyroid hormone and aldosterone [11, 34].

Little information is available on the mechanism of the effects of aldosterone in physiological concentrations on the expression of the Na,K-pump proteins in mammalian epithelia, i.e., on the transcription of the α-subunit and β-subunit genes, the specificity of the response with respect to the isoforms of the α-subunit ($\alpha 1$, $\alpha 2$, $\alpha 3$), and the interactions with other hormones. It is also important to explore the molecular mechanism behind the phenomenon of escape of the Na,K-pump from the effect of continuously elevated concentrations of aldosterone.

Here we examine the effects of aldosterone in physiological concentrations in vivo using Na^+ deprivation in rabbits to raise endogenous aldosterone secretion. Serum levels of T_3 and T_4 are monitored and the effect of replacement therapy with T_3 is examined. The expression of Na,K-pump genes is examined by qualitative analysis of α-subunit isoform distribution and quantitative analysis of the relative abundance of specific α-subunit and β-subunit mRNAs of the Na,K-pump. The total number of Na,K-pump units is determined by specific [^{3}H]-ouabain binding to the membranes of the distal colon epithelium. During biosynthesis, association between α- and β-subunit is required for proper maturation of the α-subunit and transfer of the $\alpha\beta$-unit from the endoplasmic reticulum to the plasma membrane [10]. In the regulatory conditions established in this work, both α- and β-mRNA levels are therefore monitored to see if aldosterone or thyroid hormone have differential effects on the transcription of the α- and β-subunit genes of Na,K-ATPase.

Materials and Methods

Animals and Dietary Manipulations

New Zealand White rabbits (female, approximately 2.5 kg weight) were fed either a sodium-replete diet (commercial rabbit chow containing 0.13% Na^+ and 0.8% K^+ plus tap water) or a low-sodium diet (pearl barley containing 0.003% Na^+ and 0.33% K^+ plus distilled water). The distal colon epithelium (mucosal scrapings) was prepared as previously described [43] from animals sacrificed at day 0 (n = 4), day 1 (n = 2), day 2 (n = 2), day 3 (n = 2), day 4 (n = 2), day 5 (n = 3), day 6 (n = 2), day 7 (n = 3), and day 10 (n = 2) of the low-sodium diet. Animals on day 0 of the diet regimen represent a reference group kept on a sodium-replete diet. Tissues were frozen in liquid nitrogen immediately after dissection and samples were collected and pooled from each group of animals in the diet regimen and kept at −80°C. Blood samples at the time of sacrifice (each day at 10:00 a.m.) were collected for radioimmunoassay of aldosterone (Sorin-Biomedica) and total T_3 and T_4 (Becton Dickinson) in the serum. For T_3 substitution, rabbits maintained for seven days on a low-sodium diet were injected with a single dose of T_3 (10 μg/kg of body weight subcutaneously) and killed 18 hr (n = 2) or 36 hr (n = 2) after injection.

Preparation of Crude Membrane Fractions

Aliquots of mucosal scrapings were homogenized with five strokes in a tightly fitting Teflon-glass homogenizer (Braun, Melsungen, FRG) at 1,000 rpm, in ice-cold 250 mM sucrose, 1 mM EDTA, 10 mM MOPS-Tris, pH 7.0, 0.5 g wet wt/2.5 ml. All membranes in the homogenate were sedimented in the Beckman/TL 100 ultracentrifuge for 30 min at 70,000 rpm (195,000 × g) [23]. The pellets were rehomogenized in the original volume, centrifuged again as above, resuspended in 1 mM EDTA, 10 mM MOPS-Tris, pH 7.0 to 15 mg protein/ml, and kept at −80°C. Protein was determined by the Lowry procedure as modified by Markwell [28].

RNA Extraction and Blot Analysis

Batches of total cellular RNA from distal colon epithelium (mucosal scrapings), kidney medulla, and brain were isolated by the guanidinium thiocyanate method [3]. RNA was quantified by measuring absorbance at 260 nm. Poly(A)$^+$RNA was purified by affinity chromatography on oligo(dT)-cellulose columns [18]. For Northern blotting, two micrograms of poly(A)$^+$RNA from the different tissue sources were fractionated by electrophoresis in 1% agarose/2.2 M formaldehyde [27]. The RNA was blotted onto nylon membranes (Biotrace RP, Gelman) by capillary transfer with 20 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Transfer times were 18 to 24 hr. The filters were baked at 80°C for 2 hr and then prehybridized in 50% formamide, 6 × SSC, 0.1% SDS, 5 × Denhardt's solution (0.1% polyvinyl-pyrrolidone, 0.1% Ficoll 400, 0.1% bovine serum albumin), 100 μg/ml denatured salmon DNA for 6 to 12 hr at 42°C. For slot blot analysis, total cellular RNA was denatured in 4.6 M formaldehyde, 7.5 × SSC at 65°C for 15 min and bound onto BA85 nitrocellulose filters using the Schleicher & Schuell Minifold II slot blot apparatus. The filters were baked and prehybridized as those used for Northern blots. The ^{32}P-labeled cDNA probes (*see below*) were added to the prehybridization solution and hybridized to RNA for 36 hr at 42°C. The filters were washed in 0.1 × SSC and 0.1% SDS at 50°C four times for 30 min and exposed to Fuji X-ray film at −80°C with an intensifying screen. To assure linearity of the signals in slot blot analysis, serial quantities of total RNA (2.5–10 μg) were employed and multiple exposures (17–24 hr) of autoradiograms were made. To quantify bands on the autoradiograms, we used an LKB Ultroscan XL Laser Densitometer and LKB 2400 Gelscan XL™ software. The ratio of the abundances of $\alpha 1$- and $\beta 1$-mRNA was estimated from the intensities of the resulting bands after correction for the elapsed time of exposure, length of the cDNAs and the specific activity of the respective probes.

Construction of cDNA Library and Isolation of Rabbit α-Subunit and β-Subunit cDNA Clones

For preparation of specific rabbit cDNA probes, a cDNA library was constructed using polyadenylated mRNA from the outer

renal medulla of rabbit kidney [22, 23]. mRNA was converted to double stranded DNA using reverse transcriptase and oligo(dT) as primer for the first strand synthesis and the Klenow fragment of DNA polymerase for second strand synthesis [27]. Double stranded cDNA was fractionated by electrophoresis on agarose, and cDNA in the size range of 2,000–7,000 nucleotide bases was eluted from the gel. The cDNA was supplied with EcoRI linkers and ligated into EcoRI prepared and phosphatase-treated arms of the λ ZAP II vector. Sonic extracts were used to package recombinant phages and the titer was measured using XL1 blue as host strain and color selection with IPTC/Xgal. Clones containing α-subunit and β-subunit cDNA inserts were identified by hybridization with probes of complete cDNA of the α1-subunit and β1-subunit from rat [26, 37] after ^{32}P labeling by random primed synthesis [6]. Positive rabbit cDNA clones with inserts of 2–2.4 kb were purified by repeated hybridizations and verified by DNA sequencing and alignment with rat α1-subunit [37] and β1-subunit [44] cDNA. Probes for hybridization of slot blots were prepared by excising a 1.4 kb EcoRI fragment of the rabbit α1-subunit (nt 1798–ca. 3,200 in alignment with rat α1 cDNA) and a 2.0 kb fragment of β1-subunit (nt 510–2,529 in alignment with rat β1 cDNA) that was labeled with [α-^{32}P]dATP using random primed DNA synthesis [6]. Isoform specific hybridization probes for identification of α1-, α2-, and α3-subunit and β1-subunit mRNA in Northern blots were prepared by excising DNA restriction endonuclease fragments from rat Na,K-ATPase [37] α_1 (NarI-StuI, nt 89–421), α_2 (ScaI-NheI, nt 124–498), α_3 (PstI-SmaI, nt 56–334), and β1 [44] (NcoI-StuI, nt 459–759).

[^{3}H]Ouabain Binding

For demasking latent ouabain binding sites [23], aliquots of the crude membrane fractions were incubated at 1 mg protein per ml in 0.065% deoxycholate, 10 mM MOPS-Tris, pH 7.0 for 30 min at 20°C before diluting into the [^{3}H]ouabain binding medium. Equilibrium binding was determined [22] at 0.2 mg protein per ml in the presence of increasing (0.014–1.6 μM) concentrations of [^{3}H]ouabain in (mM) 1 vanadate, 3 $MgCl_2$, 1 EDTA, 1 DTT, 10 MOPS-Tris, pH 7.0, in a total volume of 1 ml. For determination of unspecific binding, 1 mM unlabeled ouabain was added. After 1 hr incubation at 37°C the samples were cooled on ice for 15 min. Membranes were collected by vacuum filtration of 0.9 ml aliquots on 0.45 μm Millipore HA mixed cellulose ester filters and rinsed three times with 2 ml of ice-cold wash solution (mM), 1 vanadate, 3 $MgCl_2$, 1 EDTA, 1 DTT, 10 MOPS-Tris, pH 7.0. Filters were air-dried, dissolved in 3 ml Filter Count™ scintillation fluid (Packard) and counted for radioactivity by liquid scintillation spectrometry. Specific [^{3}H]ouabain binding was calculated by subtracting the unspecific binding component in the presence of 1 mM unlabeled ouabain. The data were plotted according to Scatchard and binding parameters were determined by regression analysis. Data are expressed as mean ± SE and the Student's t-test was used for statistical analysis.

Materials

[α-^{32}P] dATP (*ca.* 3,000 Ci/mmol) and [^{3}H] ouabain (18 Ci/mmol) were from Amersham. Guanidine thiocyanate and guanidine hydrochloride were from Fluka, lauryl sarcosinate (Sarkosyl® NL 30) from BDH Chemicals, Ficoll 400 and agarose from Pharmacia. Formamide from Merck was deionized by the resin AG 501-X8 from Bio-Rad. Oligo(dT) cellulose, RNA size markers, and restriction enzymes were from Boehringer Mannheim. Nylon membranes (Biotrace RP) were from Gelman. T_3 from Sigma, 2 mg T_3/ml in 0.1 N NaOH, were diluted to 0.1 μg T_3/μl in 0.005 N NaOH, 1% bovine serum albumin, pH 7.2 prior to subcutaneous application.

Results

Northern Analysis of Distal Colon Epithelium

To identify Na,K-ATPase mRNA transcripts in rabbit tissues, Northern blots of RNA were hybridized with ^{32}P-labeled isoform-specific cDNA probes prepared from α1, α2, α3, and β1 cDNA from rat. As shown in Fig. 1, only mRNA of the α1- and β1-subunits were detected in the rabbit distal colon epithelium and outer renal medulla, while mRNA of all three α-subunit isoforms (α1, α2, α3) were present in the rabbit brain. The hybridization with the α1-subunit cDNA probe revealed a single 3.7 kb mRNA in colon and brain. In brain, two mRNA species of 5.3 and 3.5 kb were detected with the α2 probe and a single 3.7 kb message with the α3 probe. In the kidney medulla a faint signal was also seen in Fig. 1 at 3.7 kb with the α3 probe. The β1 probe detected different sized mRNAs with the 2.3–2.7 kb messages being predominant. This is in agreement with the sizes of mRNA of the Na,K-ATPase subunits from other mammalian species [26]. The α1 hybridization intensities were slightly lower in the distal colon epithelium than in the brain, but considerably higher in the kidney medulla. A faint band was identified with the α2 isoform cDNA probe in the kidney medulla at 3.5 kb, but no 5.3 kb mRNA was detected. The tissue distribution of β1-mRNA resembled the pattern observed for α1-mRNA, with the highest levels present in brain and kidney.

Effect of Low-Sodium Diet on Aldosterone and Thyroid Hormone Status in Rabbits

In rabbits, the regimen of a low-sodium diet consisting of pearl barley and distilled water markedly stimulates endogenous aldosterone secretion [39]. Figure 2 shows that serum aldosterone rose nearly threefold within two days of treatment, and then continued to rise slowly to more than fourfold over most of the 10 days dietary period. We observed, however, that the diet regimen of pearl barley and distilled water led to reduced food intake and a diminution in weight gain. Such conditions can influence the thyroid hormone status of the animals [17] and

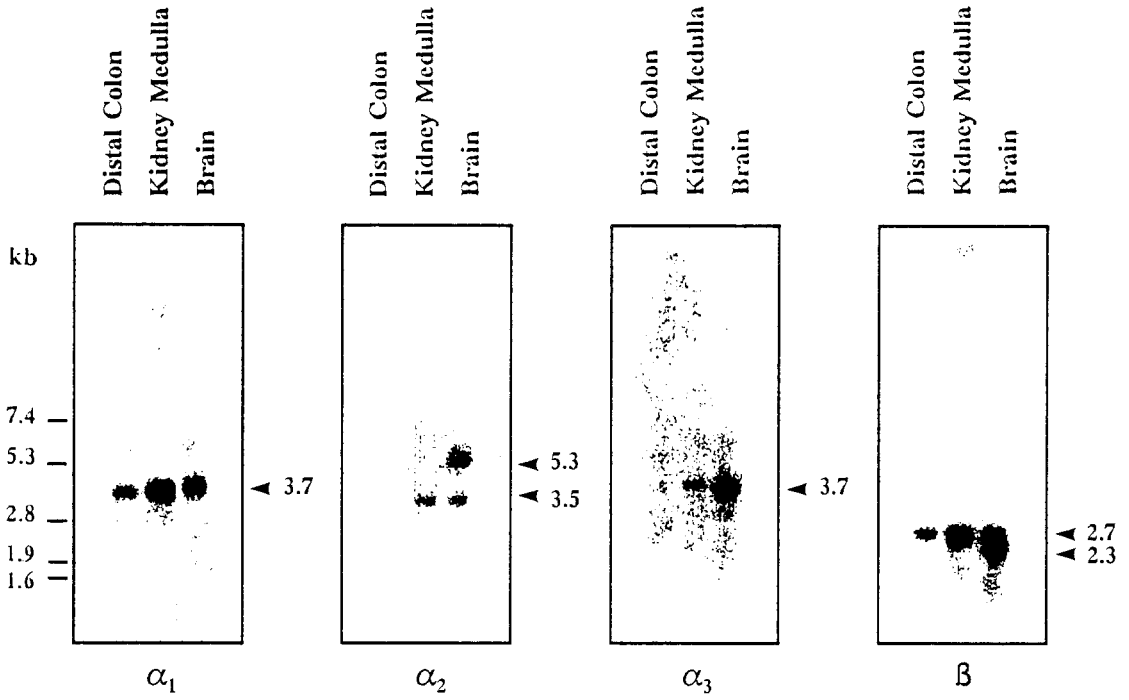

Fig. 1. RNA Northern blot analysis of Na,K-ATPase α-isoforms and β-subunit mRNAs in rabbit tissues. Poly(A)$^+$ RNA (2 μg/lane) isolated from distal colon epithelium, kidney outer medulla and brain was fractionated by electrophoresis through a 1% agarose/2.2 M formaldehyde gel, transferred to nylon membranes (Biotrace RP, Gelman), and hybridized to ^{32}P-labeled α1-, α2-, α3-, and β1-subunit cDNA probes. An autoradiogram (36 hr film exposure) for each probe is shown. An RNA ladder (4.5 μg) with the size in kb is indicated on the right of the figure.

we therefore determined the total concentrations of T_3 and T_4 in the rabbit sera. Both declined at the third day of the diet to reach a minimum level at 50–60% of control in the period from four to ten days of low-sodium diet (Fig. 2).

Na,K-ATPase α1 and β1 Subunit mRNA Abundance in Response to Low-Sodium Diet

The relative changes in α1- and β1-mRNA abundances during the 10-day dietary period were followed by RNA slot blot analysis as shown in Fig. 3. To ensure high specificity of the hybridization signals, specific rabbit α1 and β1 cDNA probes were prepared from a library of cDNA to RNA from outer renal medulla. The ratio of α1-mRNA to β1-mRNA in control conditions was estimated to be 2.7 : 1, when corrected for length and specific activities of cDNAs. A similar ratio was found in cultured kidney cells [42], while the ratio was much lower in myocardial cells [13]. Within two days of the low-sodium diet, the α1-mRNA levels increased to 162% of control while the increase of β1-mRNA was limited to 120% of control. These changes in mRNA abundance coincide with the elevation in serum aldosterone levels (*see* Fig. 2). At the third day of the diet, the decrease of T_3 and T_4 was accompanied by down-regulation of β1-mRNA to 67% and a further decrease to a level at about 55% from day 5 to day 10 of the diet. The abundance of α1-mRNA was also reduced to 63–81% of control at day 3–5, but at day 6 α1-mRNA again accumulated and rose to control values for the remaining diet period.

Ouabain Binding Capacity in Response to Low-Sodium Diet

At the protein level (Fig. 3), the concentration of Na,K-pump sites was almost doubled, to 182% of control within two days of low-sodium diet and then declined slowly, returning to the initial control values after the 10-day diet, despite the continued elevation of serum aldosterone levels (*see* Fig. 2). Analysis of the specific [^{3}H]-ouabain binding component (Fig. 4) showed that Scatchard plots of the data of control and day 2 fit straight lines in agreement with one class of binding sites with an apparent dissociation constant of K_D = 0.2 μM. Aldosterone thus increases the number of Na,K-pump sites in the

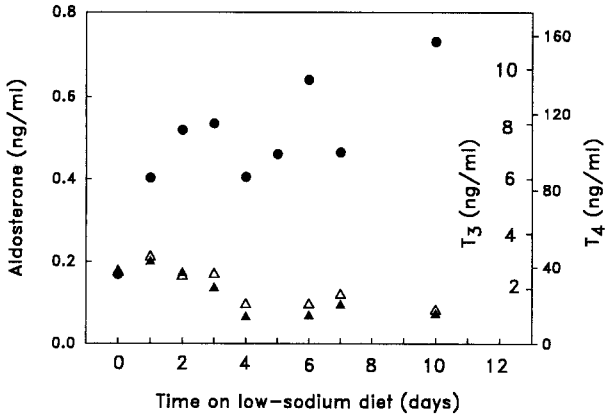

Fig. 2. Aldosterone (●) and thyroid hormone (T_3 (▲), T_4 (△)), concentrations in rabbit serum in response to a low-sodium diet. Rabbits were maintained on a low-sodium diet consisting of pearl barley and distilled water before sacrifice at the days indicated. Animals on day 0 of the diet regimen represent a reference group kept on sodium-replete diet. The data shown are the average serum concentrations of the individual hormones in ng per ml at the time of sacrifice. *See* Materials and Methods for the number of animals represented by each value.

membranes of the distal colon epithelium without altering the apparent affinity for ouabain.

Na,K-ATPase α1 and β1 Subunit mRNA and Ouabain Binding of Sodium-Deprived T_3-Injected Rabbits

Since we observed low serum concentrations of T_3 and T_4 in the rabbits kept on a low-sodium diet, a regimen of T_3 substitution was applied. In rabbits maintained for seven days on a low-sodium diet, a single subcutaneous dose of 10 μg T_3 per kg after 18 hr restored the subnormal levels of serum T_3 (1.4 ng/ml, 52% of control) to control levels (2.6 ng/ml, 96% of control). After 36 hr, serum T_3 returned to the subnormal level (1.1 ng/ml, 41% of control) seen before injection. As seen from Fig. 5, the substitution with T_3 led to rapid accumulation of β1-mRNA and upregulation of Na,K-ATPase in the distal colon epithelium. Within 18 hr after the T_3 injection, the β1-mRNA was increased by 153% from 0.6 ± 0.2 to 1.5 ± 0.2 relative to control, while the increase in α1-mRNA was only 27% in the same time period from 1.1 ± 0.1 to 1.4 ± 0.1 of control. The mRNA accumulation after T_3 injection was accompanied by a 52% increase in maximal [^{3}H]-ouabain binding from 12 pmol/mg to 18 pmol/mg protein within two days (Fig. 5, bottom panel). Again, representative data of equilibrium binding of ouabain (Fig. 4) show that T_3 has increased the number of Na,K-pump sites without altering the kinetics of ouabain binding.

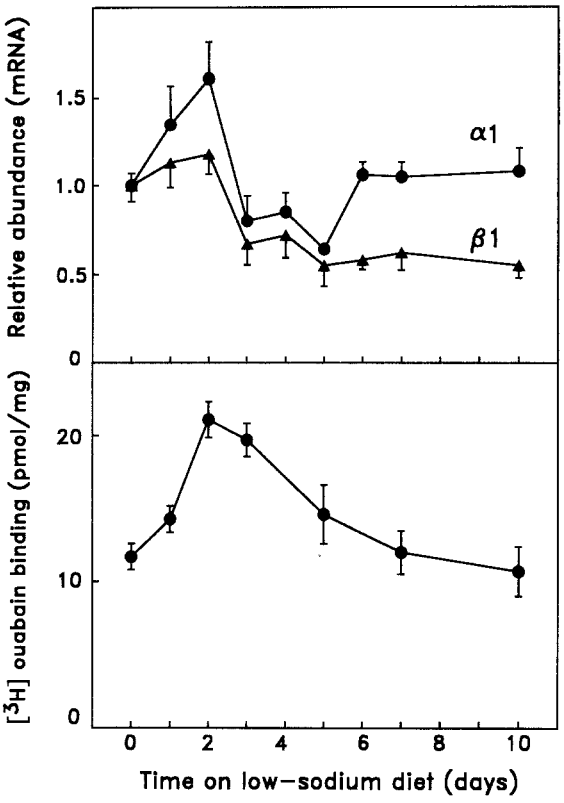

Fig. 3. Abundance of α1 (●) and β1 (▲) subunit mRNA (top panel) and specific [^{3}H]ouabain binding to membranes (bottom panel) in response to low-sodium diet. Total RNA (10 μg) from rabbit distal colon epithelium was applied to BA85 nitrocellulose filters using the Schleicher & Schuell Minifold II slot blot apparatus and hybridized with rabbit α1- and β1-subunit specific ^{32}P-labeled cDNA probes as described under Materials and Methods. The filters were autoradiographed and the hybridization intensities were determined in a densitometer. The abundance of α1-mRNA and β1-mRNA in animals maintained on the low-sodium diet for the indicated days was normalized to the mRNA abundance in the reference group (day 0) kept on the sodium-replete diet. Data are the mean ± SE (error bars) of replicate determinations from two to four different batches of RNA preparations from pooled mucosal scrapings from each treatment group (*See* Materials and Methods for the number of animals in each treatment group). For equilibrium binding, crude membrane fractions were treated with deoxycholate and incubated with 0.014–1.6 μM [^{3}H]ouabain in Mg-vanadate medium as described in Materials and Methods. Maximal [^{3}H]ouabain binding (± SE) was obtained by extrapolation of data fitted in Scatchard plots to a single-site ligand binding model.

Discussion

The data show that the abundance of α1- and β1-mRNA and the number of functional Na,K-pump units in colon surface epithelium are modulated by endogenous aldosterone and thyroid hormone in a physiological range of concentrations. The rise in

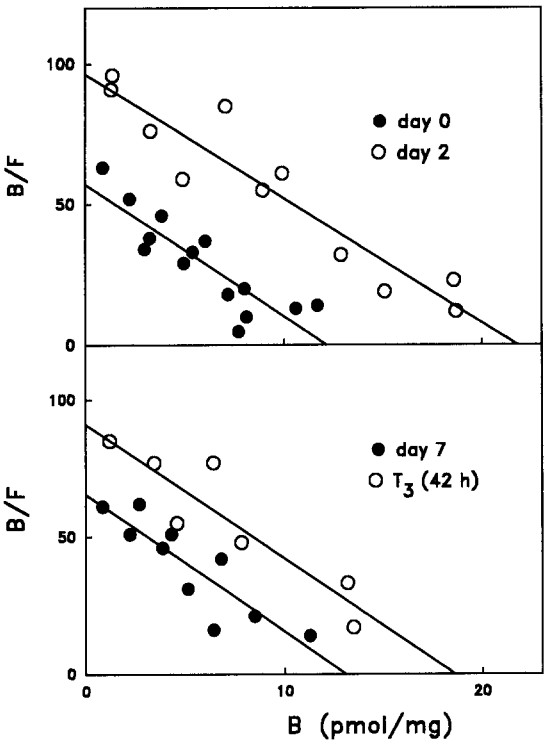

Fig. 4. Effect of sodium depletion and T_3 replacement on equilibrium binding of [^{3}H]-ouabain to membranes from rabbit distal colon epithelium. Top panel: representative Scatchard plots of specific binding in the reference group (day 0) kept on sodium-replete diet and at day 2 of low-sodium diet. B/F = bound/free; K_D = 0.2 μM. Bottom panel: plots of binding at day 7 (filled circles) and 42 hr after (open circles) injection of T_3 as in Fig. 5. B/F = bound/free; K_D = 0.2 μM.

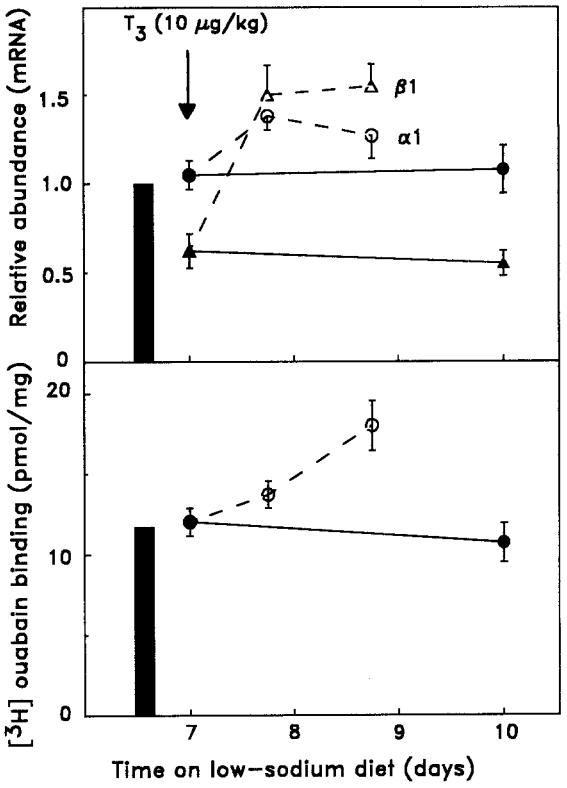

Fig. 5. Effect of T_3 injection to sodium-deprived rabbits on abundance of α1-(○) and β1-(△) mRNA (top panel) and specific [^{3}H]ouabain binding to membranes (bottom panel) in rabbit distal colon epithelium. Filled symbols represent data of untreated animals and the filled bar represents data of the reference group at day 0. Rabbits were maintained on low-sodium diet for seven days before they were injected (arrow) subcutaneously with a single dose of 10 μg T_3 per kg body weight and sacrificed 12 or 42 hr after T_3 injection. Relative abundances of α1-mRNA and β1-mRNA were determined as in Fig. 3. Values are the mean ± SE (error bars) of replicate determinations from two different batches of RNA and are normalized to reference group mRNA levels (day 0, filled bar). Maximal [^{3}H]ouabain binding (±SE) was obtained by extrapolation of data fitted in Scatchard plots to a single-site ligand binding model as in Fig. 4. The maximal binding in the reference group (day 0, filled bar) is indicated for comparison.

adrenal gland secretion leads to increments in serum aldosterone from a control level of 0.6 nM to 1.4–1.9 nM during dietary sodium deprivation. In this concentration range, the hormone effects are mediated through type I mineralocorticoid receptors ($K_{0.5}$ 0.3–3 nM), since much higher concentrations are required for binding to type II glucocorticoid receptors ($K_{0.5}$ 25–50 nM) [30]. The rise in aldosterone leads to marked accumulation of α1-mRNA, a rise of β1-mRNA, and to almost a doubling of the abundance of Na,K-pump molecules. A coordination of changes in mRNA and Na,K-pump units is observed in all our experimental conditions. During the rise in aldosterone secretion and following the drop in thyroid hormone concentration or after replacement therapy with T_3, the changes in mRNA levels precede the increment or the reduction in abundance of Na,K-pump units. This is consistent with relatively constant rates of translation of α1- or β1-mRNA. The direct effect of the hormones on the biosynthesis of the Na,K-pump can be due to altered rates of transcription of the α1 and β1 genes or to a change in stability of the mRNA species. In cardiocytes aldosterone does not alter the half-life of α1-mRNA [16]. Assays on nuclei from A6 cells demonstrate that aldosterone-(300 nM) induced increases in mRNA levels are accompanied by stimulation of the rate of transcription in a nuclear run-on assay [40].

The rise in [^{3}H]-ouabain binding sites to 182% of control is determined in a particulate fraction containing all membrane material of the surface epithelium. Since closed vesicles have been opened by detergent treatment, the data reflect changes in total number of Na,K-pump sites per cell. In the colon epithelium the relatively slow changes in concentra-

tion of [^{3}H]-ouabain binding sites per unit weight of tissue vary with the maximum capacity of the epithelium for transcellular transport of sodium in a low-sodium diet [33]. The increment in [^{3}H]-ouabain binding sites is larger when expressed in terms of sites per unit weight of epithelial tissue than in terms of sites per mg protein in preparations of basolateral membranes [33]. This is according to expectations if aldosterone increases the number of Na,K-pump sites and the membrane area in parallel with a constant density of Na,K-pump sites per unit area of basolateral membrane. In the collecting duct of rabbit kidney, the transcellular Na-flux also varies as a function of aldosterone concentration [36] in the same range as that observed here for the colon epithelium and the time course of the increment of Na,K-ATPase is correlated to the changes in basolateral membrane area [42]. Upon exposure to aldosterone no change is observed after 3–4 hr but the amplification of the basolateral membrane area begins within 24 hr and continues at a rapid rate for the first three days [42]. A similar time course is seen for the effect of deoxycorticosterone treatment on the Na,K-ATPase activity of the cortical collecting duct [32]. The increase in membrane area thus appears to be parallel to the change in number of Na,K-pump sites with a constant density of pump sites in the newly synthesized membrane. Much more rapid changes in Na,K-ATPase activity and [^{3}H]-ouabain binding capacity of the collecting duct have been observed within 3–5 hr after injection of aldosterone to adrenalectomized rabbits [1]. This fast change in activity is not accompanied by a change in membrane area [42]. Similarly, the reduced activity of Na,K-ATPase in the collecting duct of the adrenalectomized rabbit is not correlated to a reduction of basolateral membrane area [21]. The fast aldosterone-induced changes in Na,K-ATPase activity observed by Barlet and Doucet [1] in the adrenalectomized rabbit may therfore be due to mechanisms which are quite different from those causing the slow rise in concentration of Na,K-pump sites and membrane area following the increase in endogenous aldosterone secretion.

Concerning isoform and subunit specificity, our data show that elevation of aldosterone in the physiological range of concentration increases the biosynthesis of the $\alpha 1\beta 1$-isozyme of Na,K-ATPase and that the distal colon epithelium does not express the $\alpha 2$- and $\alpha 3$-isoforms. Aldosterone also induces the $\alpha 1$-isoform in A6 cells [42] and in cardiac myocytes of the rat [16]. Coordinate synthesis of the $\alpha 1$- and $\beta 1$-subunits is clearly a prerequisite for maintaining the elevated rate of Na,K-pump biosynthesis. In spite of the continued elevation of aldosterone, the number of Na,K-pump sites continued to decline in the period from day 3 to day 10 when the level of T_3 is reduced to 50%. In this period the abundance of $\beta 1$-mRNA remains at 50–60% and the reduced rate of synthesis of the $\beta 1$-subunit appears to be rate limiting for the Na,K-pump synthesis. Interrelationships between the effects of aldosterone and thyroid hormone have previously been observed. The absence of thyroid hormone greatly impairs the response of the colonic potential difference to sodium depletion or aldosterone injection [5]. A single dose of T_3 restored the aldosterone response to that found in the euthyroid state, while T_3 given without aldosterone was ineffective [5]. Thyroid hormone (T_3) also plays a permissive role in the effect of aldosterone on Na,K-ATPase activity in the collecting duct of the rabbit [1]. In the amphibian bladder T_3 antagonizes the late respone of Na,K-pump synthesis [9]. These synergisms or antagonisms can be understood as overlaps between the domains of the two hormones, but our data suggest that the explanation may rather be a different effect of aldosterone and T_3 on the transcription of the $\alpha 1$- and $\beta 1$-subunit genes of Na,K-ATPase. The α- and β-subunit depend mutually on each other for obtaining the correct configuration required for the $\alpha\beta$-unit to leave the endoplasmic reticulum to reach the Golgi and the plasma membrane [10]. Unassembled α-subunits in the endoplasmic reticulum have relatively short half-lives and the concomitant synthesis of β-subunit increases the half-life of the α-subunit. A reduction in rate of β-subunit insertion into the endoplasmic reticulum membrane following a reduction in T_3 concentration and β-mRNA will therefore reduce the half-life of the $\alpha 1$-subunit in the endoplasmic reticulum and lead to reduced overall rates of transfer of $\alpha\beta$-units to the plasma membrane. Differential regulation of Na,K-ATPase subunits by thyroid hormone has been observed in liver [2], heart, muscle and kidney [13, 15, 31].

Our data show a more pronounced effect of aldosterone on the abundance of $\alpha 1$-mRNA than on $\beta 1$-mRNA, while $\beta 1$-mRNA was reduced to lower levels than $\alpha 1$-mRNA following the reduction of T_3. After a single dose just sufficient to increase the concentration of T_3 in serum to the normal level, the magnitude of the $\beta 1$-mRNA increment far exceeded that of $\alpha 1$-mRNA. Since this therapy restored the effect of aldosterone on the amount of functional Na,K-pump sites, the data confirm that the rate of $\beta 1$-subunit synthesis is rate limiting in the period of hypothyroidism. Our data thus suggest that T_3 via its nuclear receptor system is involved in regulation of β-subunit genes in colon and that this interaction is permissive for the aldosterone effect at the transcriptional level.

The interactions of aldosterone, thyroid hor-

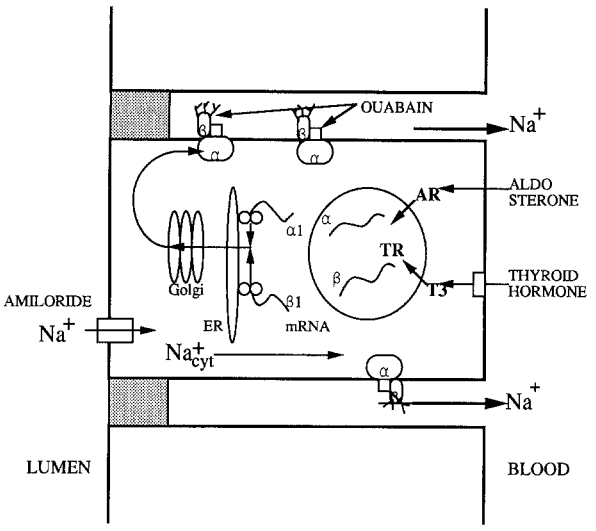

Fig. 6. Model of Na,K-pump biosynthesis illustrating the possible molecular mechanisms for escape from the effect of aldosterone on the rate of Na,K-pump biosynthesis. The α- and β-subunits emerging from the ribosomes associate in the endoplasmic reticulum (*ER*) before migration to the Golgi and the basolateral membrane. Aldosterone increases the rate of $\alpha 1\beta 1$-unit synthesis presumably through an effect on the rate of $\alpha 1$- and $\beta 1$-gene transcription. At high aldosterone, escape is seen when reduced levels of $[Na^+]_{cyt}$ and/or thyroid hormone limit the rate of β-subunit synthesis.

mone and $[Na^+]_{cyt}$ in regulation of the α- and β-subunit genes are summarized in Fig. 6, illustrating the possible molecular mechanisms for escape from the effect of aldosterone on the number of Na,K-pump sites. As pointed out by O'Neil and Hayhurst [32], low $[Na^+]_{cyt}$ levels can interrupt the aldosterone-induced rise in ouabain binding sites with return to control levels of the number of Na,K-pump sites. The morphological response to aldosterone treatment was also prevented by a low-Na diet [42]. The effect of $[Na^+]_{cyt}$ on the amount of Na,K-ATPase has been demonstrated in rat kidney [20] and a number of other tissues [32, 34] and data on cultured muscle [38] and kidney [25] cells show that changes of $[Na^+]_{cyt}$ predominantly affect the level of $\beta 1$-mRNA. However, reduction of $[Na^+]_{cyt}$ is hardly the mechanism responsible for the reduced rates of Na,K-pump biosynthesis in our experiments, since it has been shown that $[Na^+]_{cyt}$ in colon epithelium remains within the normal range in low-sodium diets [35, 39]. As shown in the present work, a low level of T_3 will suppress mRNA levels, in particular for the β-subunit, leading to Na,K-pump concentrations in the cell returning to control levels in spite of continued maintenance of aldosterone levels three to fourfold higher than control. Reduction of T_3 and/or $[Na^+]_{cyt}$ serve to limit biosynthesis of Na,K-ATPase in situations of limited energy supply or limited supply of Na^+, a substrate for the pump. They both seem to work at the level of the β-subunit gene, affecting synthesis of the β-subunit as a limiting factor for assembly of the $\alpha\beta$-unit and functional maturation of the Na,K-pump.

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