melatonin biosynthesis. A failure of the light pulse to suppress melatonin production in pineals may simply reflect differences in the accessibility of these two organs to environmental light. The higher intensity, longer duration of pulse or repeated exposure to light might suppress melatonin production in embryonic pineals as well. On the other hand, different sensitivity to light or a delayed development of photosensitive elements in pineals in comparison with eyes are also possible. In 1-day-old Japanese quail, high dark-time melatonin concentrations were suppressed to basal level 5 min after light exposure (Zeman and Gwinner, in preparation), indicating that immediately after hatching the response of the system to unexpected light already resembles the response of the mature system.

Our results suggest that the daily rhythm of melatonin production as part of the avian circadian system is developed before hatching in this precocial bird. The rhythmic melatonin production is synchronized to a light-dark cycle during incubation, and at the end of embryonic life it can respond almost instantaneously to a light pulse applied during the dark period.

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Regulation of rat renal ($Na^+ + K^+$)-adenosine triphosphatase mRNA levels by corticosterone*

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Abstract. We investigated the mechanisms for glucocorticoid regulation of rat renal NaK-ATPase activity. Our findings suggest that the magnitudes of corticosterone-induced increases in α_1 mRNA and β_1 mRNA levels are similar in the kidney of the adult adrenalectomized rats. The results also suggest that corticosterone restores NaK-ATPase activity in adrenalectomized rats prior to any enhanced sodium delivery.

Key words. Corticosterone; adrenalectomy; NaK-ATPase subunits; NaK-ATPase mRNAs; glomerular filtration rate.

($Na^+ + K^+$)-adenosine triphosphatase (NaK-ATPase, EC 3. 6. 1. 3) is an integral plasma membrane protein which maintains the electrochemical Na^+ and K^+ gradients required for many vital functions. These include the regulation of cell volume, the propagation of action potentials in excitable tissues and the active transport of nutrients. The enzyme is believed to exist as a heterodimer consisting of a catalytic α subunit and a β subunit. Both subunits are encoded by multigene families and the biochemically distinct isoforms of each subunit are differentially expressed with regard to tissue type[1-3]. Glucocorticoids increase NaK-ATPase activity in the kidney[4-6]. These hormones also increase renal blood flow and glomerular filtration rate[7]. The elevated NaK-ATPase activity in the kidney could be due to the enhanced glomerular filtration rate and filtered sodium load[8]. In isolated superfused distal tubules, glucocorticoids increase NaK-ATPase activity[9] suggesting a direct action of these hormones at the tubular epithelial cells. One objective of our investigation was to test whether a change in net Na^+ reabsorption could mediate the change in NaK-ATPase activity on treatment with corticosterone. The results indicate that corticosterone restores renal NaK-ATPase activity in adrenalectomized rats prior to any enhanced sodium delivery.

Glucocorticoid-mediated increases in renal NaK-ATPase activity and in quantities of the phosphorylated intermediate[10] suggest that these hormones mediate an enhanced NaK-ATPase activity via an increase in the number of active enzyme units. Recent studies demonstrate

that the abundance of NaK-ATPase (α_1 and β_1) mRNAs is regulated by betamethasone in the infant rat kidney (10 days old) but not in the adult rat kidney (50 days old)[11], and by dexamethasone in a rat liver cell line and liver cells[12]. Our findings indicate that corticosterone increases the levels of the NaK-ATPase subunits in the adult rats, at least in part, through increased mRNA content.

Materials and methods

Materials. The materials used for electrophoresis were purchased from Sigma. [α-^{32}P]-Cytidine 5'-triphosphate (CTP) (spec. act. 3000 Ci/mmol) and the Random Primer Extension Labeling System were purchased from DuPont/NEN Research Products. Restriction enzymes were obtained from Bethesda Research Laboratory. All chemicals and solvents used in enzyme and antibody studies were analytical reagent grade and those used in RNA studies were molecular biology grade.

Enzyme preparation. Tissue samples for enzyme assay were prepared according to Lo and Lo[13]. Male, Sprague Dawley rats (180–200 g) were surgically adrenalectomized following induction of anesthesia with chloral hydrate (36 mg/100 g b.wt, i.p.). After surgery they were maintained on rat chow (Agway 1257) ad libitum and 0.9 g% saline. Seven to ten days after surgery, corticosterone was administered s.c. in 0.5% ethanol in saline. At different time schedules, the rats were decapitated, the kidneys were removed and decapsulated, and the cortex and outer medulla were collected by gross dissection. The tissues were then homogenized in a medium containing 0.1% deoxycholate, 0.03 M D,L-histidine, 0.005 M EDTA, and 0.25 M sucrose, pH 7.4. The homogenate was centrifuged at low speed (1000 × g for 10 min) at 4 °C in a Sorvall SS-34 rotor to remove cell debris and nuclei. The supernatant was collected, stored overnight at −80 °C, and then analyzed for NaK-ATPase activity and protein.

Enzyme assay. NaK-ATPase activity was measured as the difference between inorganic phosphate released in the presence and absence of 1 mM ouabain, as previously described[14]. The reaction was started with the addition of 0.1 ml of the homogenate (25 µg protein) to 0.9 ml of medium that contained 0.1 M NaCl, 0.02 M KCl, 0.003 M $MgCl_2$, 0.1 M Tris, 0.001 M EDTA, pH 7.4, or with 10^{-3} M ouabain added. The samples were then incubated for 15 min at 37 °C after starting the reaction by the addition of 0.1 ml of 0.03 M ATP (Tris salt). These conditions provided linear rates of inorganic phosphate production for 20 min and linear increases in the generation of P_i with protein concentrations up to 50 µg of protein/ml. The reaction was terminated by the addition of 0.2 ml ice-cold 30% trichloroacetic acid and p_i was determined by the method of Fiske and Subbarow[15]. Mg-ATPase activity was assayed in the medium with added ouabain, and the NaK-ATPase activity was calculated as the difference in activity of the complete system ($+Na^+$, $+K^+$, $+Mg^{2+}$) from that in the presence of ouabain. Specific activity was expressed as micromoles of P_i released per /mg protein. Protein content was determined by the Lowry method[16].

Renal function studies. The adrenalectomized rats were divided into three groups, each representing a time point in the experiment, i.e. 0 (untreated), 12 and 24 h after corticosterone treatment (1 dose, 1 mg/100 g b.wt). A group of sham-operated animals was also studied. Following induction of anesthesia, as described above, a catheter (PE-10) filled with heparinized saline was inserted into the left carotid artery and another into the right external jugular vein. A third catheter (PE-50) was placed in the bladder. After 30 min of urine collection, 1 ml of arterial blood was collected and a priming bolus of 0.5 ml of inulin (3 mg/ml) and p-aminohippuric acid (PAH, 1 mg/ml) in heparinized saline was administered via the jugular vein. This was followed by a sustaining infusion of the same solution with a constant infusion pump (Harvard Apparatus) at a rate of 0.018 ml/min for 2 h, at which time a constant level of plasma inulin and PAH was reached. Plasma inulin and PAH concentrations in different groups of rats were similar (data not shown). Urine was collected throughout the infusion and the final volume recorded. During the final 5 min of the infusion a 1-ml blood sample was obtained. Hematocrits were determined in all the blood samples. Protein-free plasma was obtained by the addition of 0.9% $CdSO_4$ and 0.1 N NaOH (final concentration) followed by centrifugation at 10,000 g for 5 min. Sodium and potassium concentrations in the plasma and in the urine were analyzed by a Beckman Klina atomic absorption flamephotometer. The urine and plasma samples were assayed spectrophotometrically for both inulin and PAH using the anthrone method[17] and the method of Smith[18], respectively. Glomerular filtration rate and renal plasma flow were estimated from the clearance of inulin and PAH, respectively.

Quantitation of NaK-ATPase α_1 and β_1 mRNAs. Due to the rapid degradation of mRNA in isolated rat kidneys, whole kidneys were rapidly frozen in liquid nitrogen and total RNA was isolated using guanidinium thiocyanate[19]. To ensure loading of equal amounts of total RNA (10 µg per sample) to the agarose gel and transfer onto nylon membrane (Duralon-UV, Stratagene), ethidium bromide-stained 28S and 18S rRNAs were monitored in every experiment. Abundance of NaK-ATPase α_1 and β_1 mRNAs were quantitated by Northern blot analysis[20]. The hybridization was performed according to Church and Gilbert[21] at 65 °C for 12 h containing an excess of a heat-denatured, full-length rat brain NaK-ATPase α_1 or β_1 complementary DNA (Gift from Dr R. Levenson, Yale University) labeled with [α-^{32}P] (3000 C_i/mmol) using a random primer extension labeling system. In addition, the effect of corticosterone on the contents

of mRNAs (α_1 and β_1) was quantitated by slot blot analysis. Total RNA (0.1 µg/slot) was applied directly to the nylon and was prehybridized followed by hybridizing with radiolabeled cDNAs for NaK-ATPase α_1 and β_1 subunits. Autoradiography was followed by densitometric scanning of the X-ray film with a Beckman DU-8 spectrophotometer.

Results

Regulation of NaK-ATPase activities by corticosterone. The NaK-ATPase activities from the renal cortex and medulla decreased 28% and 51%, respectively, 7–10 days following adrenalectomy (SHAM vs ADX). NaK-ATPase activities reached 93% of sham-operated values in the cortex and 69% in the medulla after corticosterone (250 µg/100 g b.wt, twice daily, 3 days, i.p.) treatment to the adrenalectomized rats (fig. 1). The lack of Mg-ATPase response supports a selective enrichment of NaK-ATPase rather than a generalized plasma membrane hypertrophy.

NaK-ATPase activities in both cortical and medullary tissues from the adrenalectomized rats were significantly increased 6 h following a single injection of corticosterone (1 mg/100 g b.wt) (fig. 2). Cortical NaK-ATPase activity increased by 31% at 6 h, rising to a peak of 41% by 24 h before decreasing almost to the diluent level by 48 h. At the 6 h time point, the medullary NaK-ATPase activity was 65% greater than that seen in the diluent treated animal. The enzyme activity remained elevated through 12 h and showed a gradual decline between 12 h and 48 h after corticosterone treatment. Mg-ATPase activity was unaffected at all time points in the cortex and was only transiently enhanced (at the 12 h time point) in the medulla.

The dose dependence of NaK-ATPase activity in the cortex and the outer medulla was observed 24 h after a single injection of corticosterone (fig. 3). The increase in renal cortical NaK-ATPase activity reached 35, 48 and 55% following administration of 100, 250 and 500 µg corticosterone/100 g b.wt, respectively. In the outer medulla, the

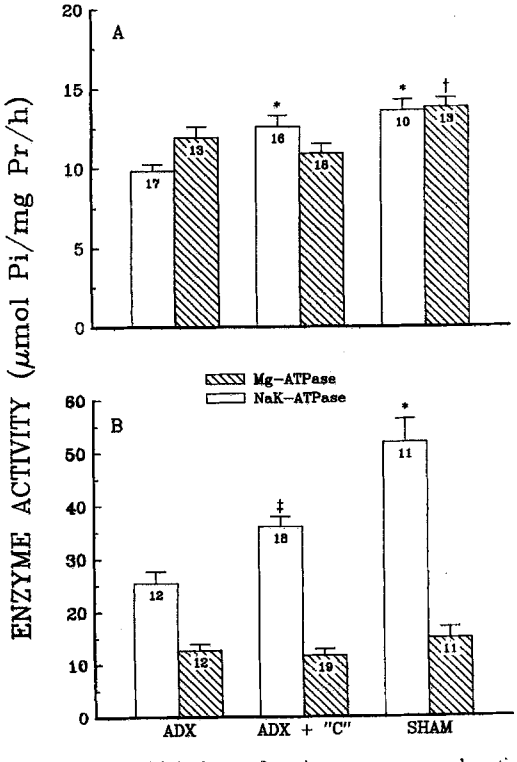

Figure 1. Effect of multiple doses of corticosterone on renal cortical and outer medullary NaK-ATPase- and Mg-ATPase specific activities. Adrenalectomized rats were injected with either diluent (ADX) or corticosterone (ADX + C) (250 µg/100 g b.wt) twice daily for 3 successive days. Sham-operated rats (SHAM) were treated with diluent according to the same schedule. The rats were killed and renal cortices (Panel A) and outer medullas (Panel B) were dissected for enzyme preparation as described in the method section. The number of observations is printed in the bars. Values are the mean ± SEM. P values are determined with the analysis of variance followed by Duncans' multiple range test.
P_i = inorganic phosphate, Pr = protein, h = hour. *$p < 0.05$ when compared with the ADX group. † $p < 0.05$ when compared with the ADX + "C". ‡ $p < 0.05$ when compared to both ADX and SHAM groups.

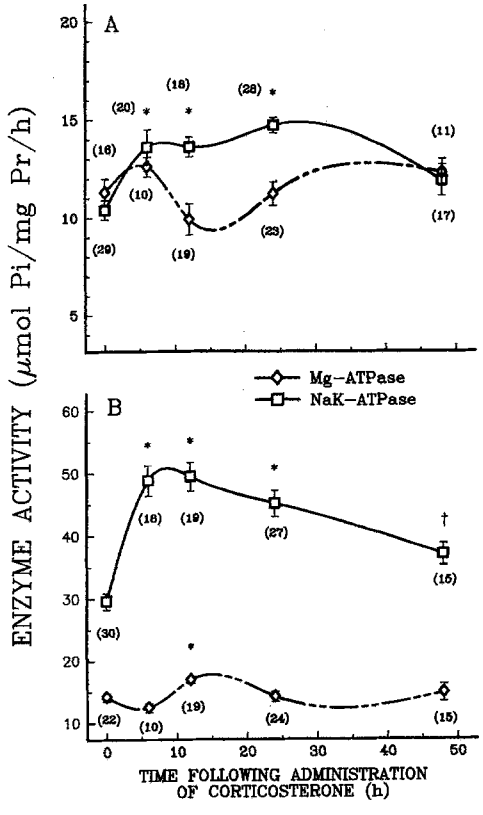

Figure 2. Temporal changes in renal cortical and outer medullary NaK-ATPase and Mg-ATPase specific activities after corticosterone. Adrenalectomized rats were administered a single dose of corticosterone (1 mg/100 g b.wt). Six, 12, 24, and 28 h, following the injection, the rats were killed and the renal cortices (Panel A) and outer medullas (Panel B) were removed for enzyme assay as described in the method section. The number of observations is parentheses. See figure 1 (legend) for statistical details.
P_i = inorganic phosphate, Pr = protein, h = hour. *$p < 0.05$ when compared to the 'diluent-treated' (zero time point) group. † $p < 0.05$ when compared to the 24 h 'corticosterone-treated' group.

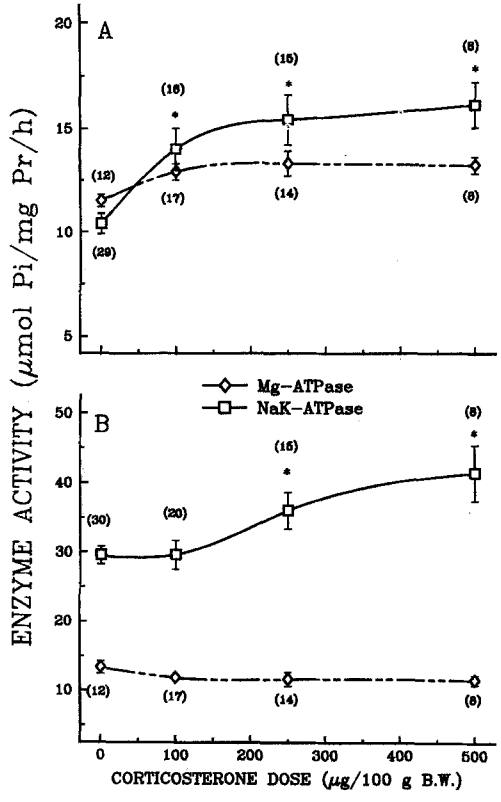

Figure 3. Dose response of renal cortical and outer medullary NaK-ATPase and Mg-ATPase specific activities after corticosterone. Adrenalectomized rats were injected with a single dose of corticosterone 24 h prior to decapitation and dissection of cortices (Panel A) and medullas (Panel B) for enzyme assay as described in the method section. The number of observations is in parentheses. See figure 1 (legend) for statistical details.
P_i = inorganic phosphate, Pr = protein, h = hour. *p < 0.05 when compared to the 'diluent-treated' group (zero dose group).

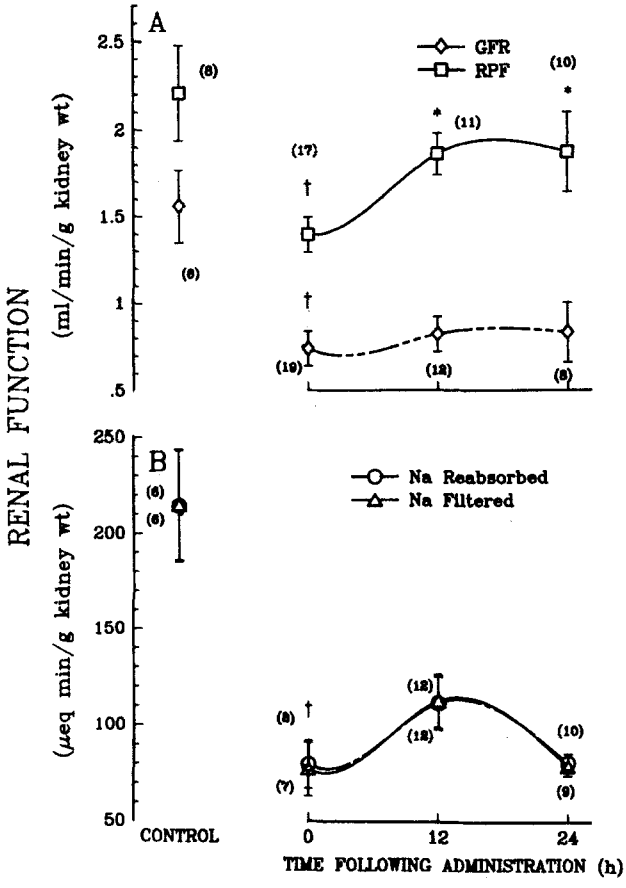

Figure 4. Temporal changes of renal functions following 7 days post-adrenalectomy and subsequent supplementation with corticosterone (1 dose, 1 mg/100 g b.wt). Experimental details were given in the method section under 'Renal function studies'. The number of observations is in parentheses. See figure 1 (legend) for statistical details. wt = weight, μeq = microequivalent, g = gram, Na = sodium, GFR = glomerular filtration rate, RPF = renal plasma flow. † p < 0.05 when compared to the 'control' group (SHAM). * p < 0.05 when compared to the 'diluent-treated' group (zero hour).

same doses enhanced NaK-ATPase activity by 0.3%, 22% and 40%, respectively. None of the above doses of corticosterone produced a change in Mg-ATPase activity in either tissue.

Renal function studies. Decrease in renal functions was observed 7 days after adrenalectomy (fig. 4). Renal plasma flow decreased by 37%, accompanied by a 52% drop in the glomerular filtration rate. Sodium filtration and reabsorption were both diminished by 63–64%. Within 12 h following administration of a single dose (1 mg/100 g b.wt) of corticosterone, renal plasma flow was essentially restored with a 33% increase and maintained at the 24 h time point. In contrast, values for glomerular filtration rate, Na^+ filtration and Na^+ reabsorption were not changed at 12 and 24 h following administration of corticosterone. Plasma p-aminohippuric acid and inulin showed no changes during the whole period of infusion (data not shown). In addition, hematocrit, plasma Na^+ and K^+ concentrations were unchanged throughout these studies (data not shown).

Effect of corticosterone on NaK-ATPase α_1 and β_1 subunit mRNA levels. Autoradiographs were produced from Northern blotted total RNA probed with α_1 and β_1 subunit cDNAs. The signals representing the α_1 and β_1 mRNA levels increased at 1 h following a single dose of corticosterone (1 mg/100 g b.wt) when compared to the adrenalectomized signals (fig. 5).These increases were more pronounced at 6 h after corticosterone treatment. The levels of α_1 and β_1 subunit mRNA observed at 6 h are the same as in the sham-operated value. In addition, autoradiographs from slot blots were prepared and scanned. After a single dose of corticosterone (1 mg/100 g b.wt) given to the adrenalectomized rats, α_1 and β_1 subunit mRNA levels increased significantly within 1 h and approaching to the levels obtained from the sham-operated rat kidneys (fig. 6).

Discussion

Mineralocorticoids have long been accepted as critical in the maintenance of sodium and water homeostasis. In Addison's disease, the concentrating and diluting capacities were shown to be alleviated by cortisone replace-

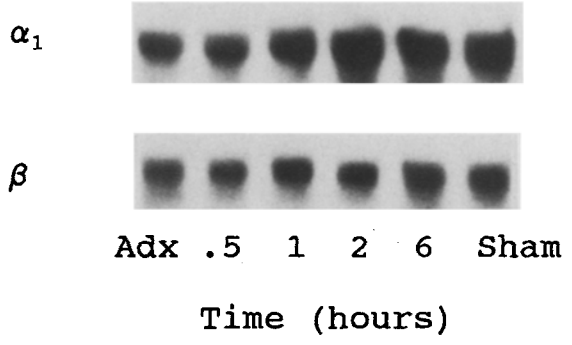

Figure 5. Time course of rat renal NaK-ATPase gene expression after corticosterone. Adrenalectomized rats were injected with corticosterone (1 mg/100 g b.wt) and the kidneys removed at 0.5, 1, 2, and 6 h after injection. Total RNA was isolated, electrophoresed (15 µg/lane), transferred to nylon membrane, and hybridized with a ^{32}P-labeled α_1 or β NaK-ATPase cDNA probe, respectively. Results of three pooled rat kidneys from each group were shown. Adx = adrenalectomized.

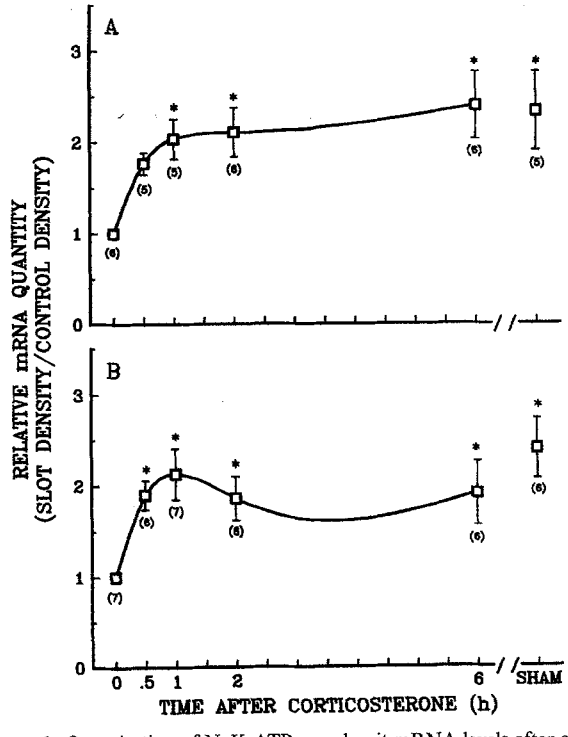

Figure 6. Quantitation of NaK-ATPase subunit mRNA levels after corticosterone. Adrenalectomized rats were injected with corticosterone (1 mg/100 g b.wt) and the kidneys removed at 0.5, 1, 2, and 6 h after injection. Total RNA was isolated and blotted directly onto the nylon membrane. Autoradiographs were prepared from the slot blots containing total kidney RNA (0.1 µg/slot). The blots were hybridized with random primer ^{32}P-labeled α_1 (Panel A) and β_1 (Panel B) cDNAs and were scanned with a spectrophotometer. Signals from different experiments were normalized to their own control (zero h) values. The number of observations is in parentheses. See figure 1 (legend) for statistical details. *$p < 0.05$ when compared to the 'zero hour' group.

ment[22] but not by aldosterone[23]. NaK-ATPase activity has been shown to rise rapidly in the thick ascending limb of weaning rats (16–20 days), coincident with a surge in serum corticosterone and an increase in urinary concentrating capacity[24]. Adrenalectomy, prior to this developmental stage, abolished both the enhanced enzyme activity and the renal functional development. Dexamethasone enhances NaK-ATPase activity notably in the medullary thick ascending limb of the loop of Henle[25]. Our results suggest that corticosterone enhances renal cortical NaK-ATPase activities but more so in renal medullary NaK-ATPase activities (fig. 2) 6 h after one dose of corticosterone given to the adrenalectomized rats.

Glucocorticoids have been demonstrated to restore NaK-ATPase activity in isolated renal tubular segments[9], in the heart[26] and in the submandibular gland[27], suggesting that this regulation has critical implications in the study of renal function and whole animal physiology.

The present studies demonstrate that a single dose of corticosterone enhances NaK-ATPase activity in the rat renal cortex and outer medulla within 6 h (fig. 2) while having no effect on glomerular filtration rate, sodium filtered, and sodium reabsorption within the first 24 h (fig. 4). The increase in renal plasma flow 12 h after corticosterone treatment could be due to the dilation of afferent and efferent arterioles which would not result in increased glomerular filtration rate. These findings are consistent with previous evidence of glucocorticoid modulation through a mechanism independent of changes in renal function[28]. A direct effect was further supported by investigations with isolated, superfused renal tubules in which the addition of dexamethasone augmented NaK-ATPase activity[9].

Kinetic studies revealed that dexamethasone administration to adrenalectomized rats increased V_{max} for Na^+, K^+ and ATP but the apparent affinities ($K_{1/2}$) for all three substrates were not affected[10,29]. Glucocorticoids also augmented the quantities of the phosphorylated intermediate[6]. The increases in V_{max} for Na^+, K^+, and ATP and in the phosphorylated intermediate indicate that glucocorticoids increase the number of NaK-ATPase units implying augmentation of synthesis. We, therefore, studied the effect of corticosterone on NaK-ATPase subunit mRNA levels.

NaK-ATPase activity can be directly regulated by ligands (e.g. Na^+, K^+, ATP, cardiac glycosides) or indirectly by hormones and growth factors. The time response of NaK-ATPase to various hormones and growth factors varies from minutes to hours and days[30,31]. Previously, we demonstrated that renal cortical NaK-ATPase activity shows no change at 12 h and progressively increases to a peak of +83% at 48 h in response to thyroid hormone[14]. Injection of triiodothyronine augments α_1 mRNA content by 2.1- and 2.5-fold in kidney cortex at 24 h and 72 h, respectively[32].

Our results demonstrated that NaK-ATPase activities increased significantly at 6 h in response to corticosterone (fig. 2). In addition, NaK-ATPase α_1 and β_1 mRNA contents were significantly increased to a peak at 1 h after corticosterone injection (fig. 6). Although no studies were conducted on the effect of corticosterone on the

turn-over of this enzyme, our results demonstrate that NaK-ATPase activity and NaK-ATPase mRNA levels increase much more rapidly in the corticosterone-treated rats (figs 2 and 6) than in the triiodothyronine-treated rats[14,32]. It is possible that the corticosterone-receptor complex reaches the specific DNA binding site sooner than the triiodothyronine-receptor complex.

Glucocorticoids are known to enhance gene expression through interaction of DNA binding proteins with specific gene regulatory regions[33]. They also modulate target mRNA stability[34]. Northern blot and slot blot analysis provided evidence of a glucocorticoid-mediated increase in NaK-ATPase subunit mRNA levels (figs 5 and 6). These observations are contrary to a report[11] which indicated that betamethasone, a glucocorticoid, showed no effect on NaK-ATPase mRNA levels in adult rat kidney. Recently, there have been reports suggesting that dexamethasone induces a disproportionately larger increase in β_1 subunit mRNA ($>$ 35-fold) than in α_1 subunit mRNA ($\simeq$ 2-fold) in the rat liver cell line and in the liver of adrenalectomized rats[12]. Our findings suggest that the magnitudes of corticosterone-induced increases are similar in α_1 (2.3-fold increase) and β_1 (2-fold increase) subunit mRNA levels in the kidney of the adrenalectomized rat (fig. 6). Previously, we compared the effect of triiodothyronine on incorporation of methionine, fucose, and glucosamine into the α and β subunits of rat renal cortical NaK-ATPase. We observed proportionate increases (range 43%–59%) in the α and β NaK-ATPase subunits[35–37]. It is possible that dexamethasone, betamethasone, and corticosterone act differently on NaK-ATPase subunit mRNA levels in different organs or cell types.

In summary, we have demonstrated that corticosterone restores renal NaK-ATPase activity in adrenalectomized rats prior to any enhanced sodium delivery. Corticosterone augments proportionately the levels of NaK-ATPase α_1 and β_1 mRNA levels. A plausible inference is that corticosterone regulates the synthesis of the NaK-ATPase α_1 and β_1 mRNA levels by increasing the rate of gene transcription and/or by stabilizing the mRNA. Both these mechanisms will be examined in order to further understand the mechanism of actions of glucocorticoids on the NaK-ATPase system.

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