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Glucocorticoids Differentially Regulate the mRNA for Na^+,K^+-ATPase Isoforms in Infant Rat Heart

ZHENG-MING WANG AND GIANNI CELSI

Department of Pediatrics, St. Göran's Children's Hospital, Karolinska Institutet, Stockholm, Sweden

ABSTRACT. The postnatal maturation of Na^+,K^+-ATPase α- and β-subunit genes can be accelerated in the rat kidney by the administration of glucocorticoid hormones (GC). In heart, Na^+,K^+-ATPase α-isoform and β-subunit genes exhibit a complex pattern of expression during development. This study examines the role of GC in the regulation of Na^+,K^+-ATPase mRNA abundance in rat heart during infancy. In 10-d-old rats given injections with a single intraperitoneal dose of betamethasone or diluent, the Na^+,K^+-ATPase activity was 2-fold higher in treated than in control rats after 24 h. GC differentially regulated the mRNA for Na^+,K^+-ATPase subunits. A significant increase in Na^+,K^+-ATPase mRNA occurred with a dose of 2.5 μg betamethasone/100 g body weight. The following experiments were performed with a saturating dose of 60 μg betamethasone/100 g body weight. The α1 mRNA was moderately but significantly increased (1.5-fold) 6 h after treatment. The mRNA for the α2 subunit increased 2.2-fold after betamethasone treatment. The mRNA for β1 was numerically increased after 20 min (1.3-fold); it was 1.5-fold higher ($p < 0.05$) after 1 h and was 3-fold higher after 6 h ($p < 0.01$). Betamethasone treatment did not significantly change the abundance of the mRNA for the α3 subunit. The expression of actin mRNA was not altered after GC. These data indicate that GC hormones may act as a "molecular switch" in the developmental expression of the mRNA for the Na^+,K^+-ATPase α-isoforms and contribute in stimulating the maturation of rat heart during the preweaning period. (*Pediatr Res* 33: 1–4, 1993)

Abbreviations

GC, glucocorticoid hormone
SSPE, sodium chloride, sodium phosphate, EDTA

Na^+,K^+-ATPase is an integral membrane enzyme that is essential for the establishment and maintenance of the Na^+ and K^+ gradient across the cell membrane. In transporting epithelia, Na^+,K^+-ATPase is located in the basolateral membrane enabling vectorial transport of water and ions (1). In electrically excitable tissues such as nerve and muscle, Na^+,K^+-ATPase is responsible for restoration of the action potential (2). The enzyme consists of two subunits, α and β, which are encoded by a multigene family located on different chromosomes (3, 4). The catalytic α-subunit contains the intracellular binding sites for substrates and the extracellular cardiac glycosides binding region; the function of the smaller β-subunit remains unknown, but a full reconstitution of the vectorial function of Na^+,K^+-ATPase requires both α and β subunits in a 1:1 stoichiometry.

The three known isoforms of the α subunit exhibit differences in tissue specificity, developmental expression patterns, hormonal regulation, Na^+ sensitivity, and cardiac glycoside affinity (5).

In rat cardiac tissue, there is evidence that Na^+,K^+-ATPase isozyme expression changes during postnatal maturation. The α1 isoform, which has a low affinity for cardiac glycosides, predominates in the rat heart throughout all stages of development, both at the mRNA level (6) and at the protein level (7). A reciprocal switch in the mRNA and protein levels of α2 and α3, which have a high affinity for cardiac glycosides, was shown to occur in the preweaning period.

In the preweaning rat, during the 2nd to 3rd wk of life, an upsurge of circulating GC occurs (8, 9). This hormonal upsurge induces a rapid maturation of Na^+,K^+-ATPase in transporting epithelia, such as in the kidney and the intestine (10, 11). There is indirect evidence that this effect is due to direct transcriptional regulation of Na^+,K^+-ATPase genes, because a single injection of betamethasone in infant rats induces a rapid and coordinated increase in renal Na^+,K^+-ATPase α1 and β1 mRNA (12). In cultured neonatal rat cardiac myocytes, it has been shown that GC can up-regulate α2 mRNA and repress the inductive effect of thyroid hormones on α3 mRNA (13). However, the role of GC in the maturation of cardiac Na^+,K^+-ATPase *in vivo* is unknown.

In the present study, we therefore consider whether GC can regulate the maturation of cardiac Na^+,K^+-ATPase *in vivo*. Infant rats were treated with a single injection of betamethasone at the age of 10 d, *i.e.* before the natural upsurge of circulating GC hormones took place.

MATERIAL AND METHODS

Animals. Experiments were performed in male Sprague-Dawley rats aged 10 d (ALAB, Sollentuna, Sweden). The rats were given injections of betamethasone (60 μg/100 body weight intraperitoneally; Glaxo Labs, Ltd., Greenford, England) or vehicle at different times before anesthesia with thiobutabarbital (8 mg/100 g body weight). In one protocol, rats were treated with various doses of betamethasone, and the hearts were studied 6 h after injection. In each animal, the heart was immediately removed and homogenized.

Northern and dot blots. Total cellular RNA was isolated from the tissues, and the integrity of the RNA was evaluated in Northern blots (Fig. 1), as previously described (12). To quantify the mRNA levels, 8 μg of total RNA were denatured in ice-cold 10 mM NaOH and blotted under vacuum onto a Hybond+ nylon filter (Amersham, Buckinghamshire, UK) with a Bio-Blot SF unit (Bio-Rad Laboratories, Richmond, CA) as previously described (14). Prehybridization (20 min) and hybridization (3

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Correspondence: Gianni Celsi, M.D., Ph.D., Department of Pediatrics, St. Göran's Children's Hospital, 11281 Stockholm, Sweden.
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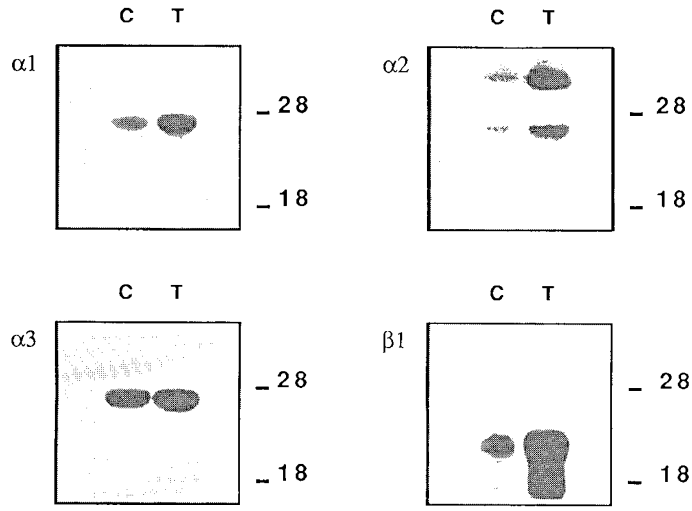

Fig. 1. Representative Northern blot analysis of hearts from 10-d-old rats. Rats were given injections of diluent (*C*) or betamethasone (*T*) (60 μg/100 g body weight) 6 h before the experiment. Positions of ribosomal 18S and 28S are indicated.

h) were performed at 65°C in Amersham Rapid Hybridization buffer. About 10^6 dpm of [^{32}P]cDNA were added for the hybridization step. The cDNA probes were random primed (Multiprime DNA labeling system, Amersham) with [α-^{32}P]cDNA to approximately the same sp act ($1–2 \times 10^9$ dpm/μg cDNA). After hybridization, the filters were washed twice with 2 × SSPE and 0.1% SDS (10 min at room temperature), once in 1 × SSPE, 0.1% SDS (15 min at 65°C), and, finally, three times in 0.1 × SSPE, 0.1% SDS (15 min each at 65°C). Autoradiographs were obtained by exposing the filters to Hyperfilm (Amersham) with two intensifying screens at −80°C. Multiple exposures of autoradiograms were made to ensure that signals were within the linear range of the film. Quantitative loading of mRNA samples was evaluated by monitoring the expression of actin mRNA for each blot. In all cases, triplicate RNA samples from experimental and control groups were analyzed simultaneously on the same nylon filter. Autoradiogram bands were quantified with an LKB Ultrascan XL laser densitometer interfaced to an IBM PC. Peaks were integrated using LKB software. Each autoradiogram was scanned three times, and the mean was calculated and normalized to an internal standard (pooled mRNA from control rats) to which an arbitrary value of 1 is given. The subunits α1 and α2 are full-length cDNA, α3 is *Pst*I-*Sma*I fragment (278 bp), and β1 is the *Hin*dIII-*Pst*I fragment (271 bp) (3, 4). Actin cDNA was purchased from Clontech Laboratories, Palo Alto, CA.

Na^+,K^+-ATPase activity assay. Enzymatic activity was determined in microsomes prepared as previously described (14). Briefly, tissues were homogenized on ice in 250 mM sucrose and 30 mM histidine buffer, pH 7.2, and centrifuged at 6 000 × *g* for 15 min at 3°C. The pellet was resuspended in the same buffer and centrifuged at 6 000 × *g* for 15 min. The supernatants from the first and the second centrifugations were removed and centrifuged at 48 000 × *g* for 30 min. After high-speed centrifugation, the supernatant was discharged and the pellet was resuspended in sucrose buffer and used for enzymatic assay.

The Na^+,K^+-ATPase activity was measured by the hydrolysis of [γ-^{32}P]ATP as previously described (12). Aliquots of proteins were preincubated for 30 min at room temperature with sodium deoxycholate (80 μg/mg protein) at a concentration predetermined to yield maximal activation of Na^+,K^+-ATPase. They were then incubated for 15 min at 37°C in 90 μL of medium containing (in mM) 115 NaCl, 10 KCl, 5 $MgCl_2$, 1 EGTA, 30 Tris-HCl, 3 Na_2 ATP, as well as a tracer amount of [γ-^{32}P]ATP, pH 7.4. The reaction was arrested by adding 700 μL of activated charcoal. The samples were left on ice for 60 min and then centrifuged in a table centrifuge. The phosphate liberated by hydrolysis of [γ-^{32}P]ATP was determined in the supernatant. Mg^{2+}-ATPase activity was determined in the presence of 1 mM ouabain and in the absence of Na^+ and K^+. Na^+,K^+-ATPase activity was calculated as the difference between total ATPase and Mg^{2+}-ATPase. In each sample, the activity was measured twice.

The protein concentration was determined by Lowry's method.

Statistical analysis. The data are presented as means ± SEM. Statistical analysis was performed with the unpaired *t* test.

RESULTS

The Na^+,K^+-ATPase activity in heart from 10-d-old rats showed a significant 2-fold increase 24 h after a single injection (60 μg/100 g body weight) of betamethasone (Fig. 2). Mg^{2+}-ATPase did not change after the betamethasone injection (7.7 ± 0.6 μmol inorganic phosphate/mg protein/h in control rats and 7.5 ± 0.6 μmol inorganic phosphate/mg protein/h in treated rats).

Six h after a single injection, betamethasone increased Na^+K^+-ATPase mRNA, and the effect was dose dependent (Fig. 3). A dose of 1 μg/100 g body weight did not induce a significant increase in β1 mRNA abundance. A significant increase occurred with 2.5 μg/100 g body weight ($p < 0.05$). A maximal response was reached at 60 μg/100 g body weight ($p < 0.01$). A dose as high as 1 000 μg/100 g body weight did not induce any further increase (no significant difference as compared with 60 μg/100 g body weight). The following experiments were therefore performed with a saturating dose (60 μg/100 g body weight). The effect of a single injection of betamethasone on the mRNA for different Na^+,K^+-ATPase isoforms is illustrated in Figures 1 and 4. A moderate but significant increase (1.5-fold) in α1 mRNA was seen in hearts from 10-d-old rats 6 h after the betamethasone injection. The mRNA for the α2 isoform was very low in control rats and increased 2.2-fold after the betamethasone injection. The mRNA for β1 increased 2.8- ± 0.3-fold. A numerical but not significant increase was observed 20 min after injection (Fig. 5). One h after injection, β1 mRNA was, significantly, 1.5-fold higher than in control rats ($p < 0.05$). The betamethasone injection did not significantly change the abundance of mRNA for the α3 subunit (Fig. 4). Because of differences in probe length, sp act, and exposure durations, the relative amounts of each isoform from the same RNA sample cannot be extrapolated from Figure 1.

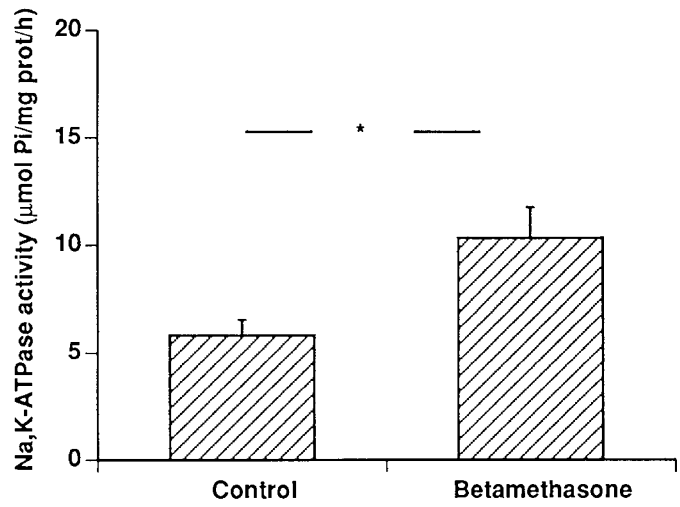

Fig. 2. Effect of betamethasone injection on Na^+,K^+-ATPase activity in heart from 10-d-old rats. Rats were given injections of diluent or betamethasone (60 μg/100 g body weight) 24 h before the experiment ($n = 6$ in each group). * indicates significant difference between groups ($p < 0.01$). *Pi*, inorganic phosphate.

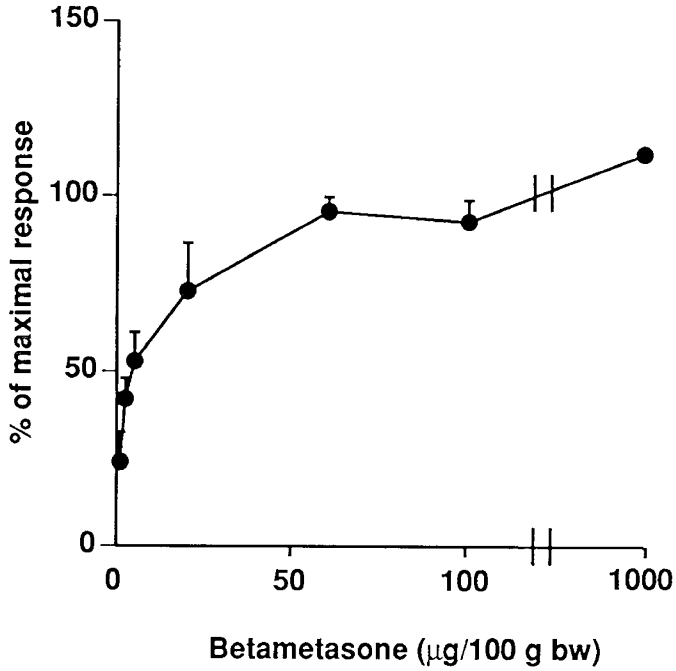

Fig. 3. Effect of different doses of betamethasone on Na^+,K^+-ATPase $\beta 1$ isoform mRNA in heart from 10-d-old rats. Rats were studied 6 h after injection ($n = 3$ in each group). An arbitrary value of 1 has been given to an internal standard (mRNA pooled from control rats).

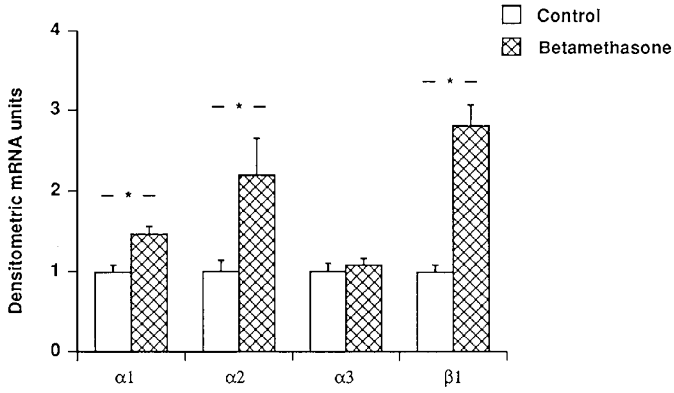

Fig. 4. Effect of betamethasone injection on mRNA abundance for different isoforms of Na^+,K^+-ATPase in hearts from 10-d-old rats. Rats were given injections of betamethasone (60 μg/100 g body weight) 6 h before the experiment ($n = 6$ in each group). An arbitrary value of 1 has been given to an internal standard (mRNA pooled from control rats). * indicates significant difference as compared with control rats ($p < 0.01$).

Actin mRNA abundance was not altered by the betamethasone injection. Therefore, even when corrected by actin mRNA abundance, the relative increase in Na^+,K^+-ATPase mRNA after the betamethasone injection did not change (data not shown).

DISCUSSION

The results of the present study indicate that GC can regulate *in vivo* the mRNA expression and the activity of cardiac Na^+,K^+-ATPase in the infant rat. A saturating dose of betamethasone, a synthetic highly specific GC, induced a 2-fold increase in enzyme activity and differentially regulated the mRNA for the Na^+,K^+-ATPase subunits. The maximal increase was observed for $\beta 1$ mRNA. Among the catalytic isoforms, $\alpha 2$ mRNA was markedly up-regulated and $\alpha 1$ mRNA was moderately increased, whereas no change in $\alpha 3$ mRNA was observed. The α and β subunit mRNA were stimulated by GC with a 1:1 stoichiometry. Taken

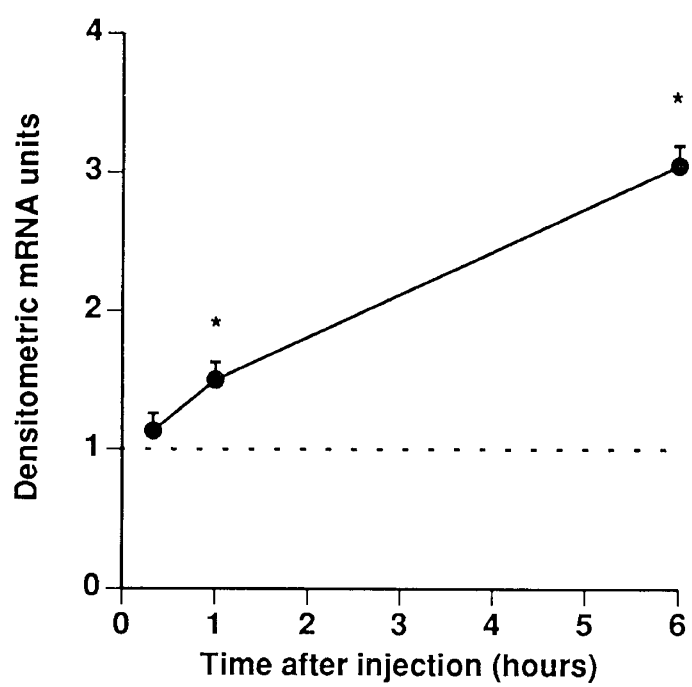

Fig. 5. Time-dependent effect of betamethasone on Na^+,K^+-ATPase $\beta 1$-isoform mRNA in heart from 10-d-old rats. Rats were given injections of betamethasone (60 μg/100 g body weight) ($n = 3$ in each group). An arbitrary value of 1 has been given to an internal standard (mRNA pooled from control rats). *Dotted line* indicates mean value in control animals. * indicates significant difference as compared with control rats.

together, these data suggest that functional Na^+,K^+-ATPase enzyme units are assembled in the infant heart after GC administration. Rats were treated with 60 μg of betamethasone/100 g body weight. This dose has been shown to have a maximal inductive effect on renal cortical enzymes taken from infant rats (15). We noted a significant increase in Na^+,K^+-ATPase mRNA with 2.5 μg of betamethasone/100 g body weight, a dose that is thought to provide a physiologic replacement in adrenalectomized animals (16). This is also the threshold dose for stimulation of lung surfactant production in near-term rats (17).

In rats, the activity of Na^+,K^+-ATPase increases during postnatal life in most tissues (9, 10, 18), including the heart (19). The expression of the different isoforms of Na^+,K^+-ATPase α and β subunits is ontogenically regulated. Among the isoforms of the catalytic α subunit, the $\alpha 1$ isoform predominates in the rat heart throughout all stages of development (7). In rats, mRNA of the $\alpha 1$ subunits has been shown to decrease (6) or to increase during postnatal life (20). In the neonatal heart, the $\alpha 3$ isoform is also expressed. A reciprocal switch in $\alpha 3$ and $\alpha 2$ isoforms occurs in the preweaning period, so that in the adult heart $\alpha 2$ is expressed but $\alpha 3$ is not (6, 7, 20). The $\beta 1$ mRNA is expressed at the same level in neonatal and adult life, with a transient peak occurring about the time of weaning (6).

The isoforms of the catalytic α subunit show differences in hormonal regulation and affinity for substrates (Na^+,K^+ and ATP) and for specific inhibitors, the cardiac glycosides (5). Although the physiologic significance of the changes occurring in the expression of cardiac Na^+,K^+-ATPase isoforms during the preweaning period remains to be evaluated (7), it is likely that the Na^+,K^+-ATPase isoforms play an important role in regulating the different myocardial inotropy and sensitivity to digitalis that are observed during infancy (21). The mechanisms that regulate Na^+,K^+-ATPase expression during cardiac maturation are still poorly understood (13, 22). In the rat, the upsurge in circulating GC that occurs during the preweaning period induces enzyme maturation in several organs, such as the intestine and the liver (23). Precocious administration of GC induces maturation of

Na^+,K^+-ATPase activity as well as fluid transport capacity in the kidney and intestine (10, 11, 24). Theoretically, the hormones could increase mRNA abundance by enhancing transcription rate or by altering RNA stability or degradation. GC are known to activate gene transcription by binding, together with the GC receptor, to specific DNA sequences on the promoter region of target genes, the GC-responsive elements (25). GC-responsive elements are present on the promoter region of Na^+,K^+-ATPase genes (26–28). We have recently suggested that GC up-regulate Na^+,K^+-ATPase mRNA in the kidney and lung only during certain periods of infancy (29), possibly throughout direct transcriptional regulation of Na^+,K^+-ATPase genes (12). The finding that cardiac Na^+,K^+-ATPase mRNA is rapidly increased after a single injection of GC also suggests that the hormones activate gene transcription. Our data, however, cannot exclude an effect also on mRNA stability.

The results of the present study strongly suggest that GC act as a "molecular switch" to stimulate the terminal differentiation of cardiac Na^+,K^+-ATPase activity by up-regulating the $\alpha 1$, $\alpha 2$, and $\beta 1$ mRNA in the preweaning period. Worthy of note is the recent report that, in the heart of young, spontaneously hypertensive rats, the Na^+,K^+-ATPase genes reach terminal differentiation at an earlier phase than they do in normotensive Wistar-Kyoto rats (20). Moreover, circulating levels of GC seem to be higher in spontaneously hypertensive rats than in Wistar-Kyoto rats during the preweaning period (30).

In addition to GC, other transcriptional factors may be of importance for the maturation of cardiac Na^+,K^+-ATPase. It has been shown that, in cultures of rat neonatal cardiocytes, thyroid hormone can increase $\alpha 2$, $\alpha 3$, and $\beta 1$ mRNA, and dexamethasone can up-regulate $\alpha 2$ mRNA and repress the inductive effect of thyroid hormones on $\alpha 3$ mRNA (13). However, prenatal thyroidectomy did not alter the expression of cardiac Na^+,K^+-ATPase isoforms in neonatal and infant rats (22), nor did thyroid hormone treatment increase the $\alpha 2$ mRNA abundance in infant rat heart (31). This should be an important topic for further studies to characterize *in vivo* the transcriptional factors that interact with GC for regulating the maturation of the different isoforms of Na^+,K^+-ATPase in the infant heart.

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REFERENCES

1. Jörgensen PL 1986 Structure, function and regulation of Na^+,K^+-ATPase in the kidney. Kidney Int 29:10–20
2. Smith TH 1988 Digitalis: mechanisms of action and clinical use. N Engl J Med 318:358–365
3. Shull GE, Greeb J, Lingrel JB 1986 Molecular cloning of three distinct forms of the Na^+,K^+-ATPase α-subunit from rat brain. Biochemistry 25:8125–8132
4. Young RM, Shull GE, Lingrel JB 1987 Multiple mRNAs from rat kidney and brain encode a single Na^+,K^+-ATPase β subunit protein. J Biol Chem 262:4905–4910
5. Sweadner KJ 1989 Isozymes of the Na^+/K^+-ATPase. Biochim Biophys Acta 988:185–220
6. Orlowski J, Lingrel JB 1988 Tissue-specific and developmental regulation of rat Na^+,K^+-ATPase catalytic α isoform and β subunit mRNAs. J Biol Chem 263:10 436–10 442
7. Lucchesi PA, Sweadner KJ 1991 Postnatal changes in Na, K-ATPase isoform expression in rat cardiac ventricle. J Biol Chem 266:9327–9331
8. Henning SJ 1978 Plasma concentrations of total and free corticosterone during development in the rat. Am J Physiol 235:E451–E456
9. Celsi G, Larsson L, Aperia A 1986 Proximal tubular reabsorption and Na-K-ATPase activity in remnant kidney of young rats. Am J Physiol 251:F588–F593
10. Finkel Y, Aperia A 1986 Role of aldosterone for control of colonic NaK-ATPase activity in weanling rats. Pediatr Res 20:242–245
11. Rane S, Aperia A 1985 Ontogeny of Na-K-ATPase activity in thick ascending limb and of concentrating capacity. Am J Physiol 249:F723–F728
12. Celsi G, Nishi A, Akusjärvi G, Aperia A 1991 Abundance of Na^+-K^+-ATPase mRNA is regulated by glucocorticoid hormones in infant rat kidneys. Am J Physiol 260:F192–F197
13. Orlowski J, Lingrel JB 1990 Thyroid and glucocorticoid hormones regulate the expression of multiple Na,K-ATPase genes in cultured neonatal rat cardiac myocytes. J Biol Chem 265:3462–3470
14. Celsi G, Stålh J, Wang Z-M, Nishi A 1992 Adreno-corticoid regulation of Na^+,K^+-ATPase in adult rat kidney: effects on posttranslational processing and mRNA abundance. Acta Physiol Scand 145:85–91
15. Igarashi Y, Aperia A, Larsson L, Zetterström R 1983 Effect of betamethasone on Na-K-ATPase activity and basal and lateral cell membrane in proximal tubular cells during early development. Am J Physiol 245:F232–F237
16. Stanton B, Klein-Robbenhaar G, Wade J, Giebisch G, DeFronzo R 1985 Effects of adrenalectomy and chronic adrenal corticosteroid replacement on potassium transport in rat kidney. J Clin Invest 75:1317–1326
17. Anderson GG, Lamden M, Cidlowski JA, Ashikaga T 1981 Comparative pulmonary surfactant-inducing effect of three corticosteroids in the near-term rat. Am J Obstet Gynecol 139:562–564
18. Schmitt CA, McDonough AA 1986 Developmental and thyroid hormone regulation of two molecular forms of Na^+-K^+-ATPase in brain. J Biol Chem 261:10 439–10 444
19. Charlemagne D, Maixent J-M, Preteseille M, Lelievre LG 1986 Ouabain binding sites and (Na+,K+)-ATPase activity in rat cardiac hypertrophy. Expression of the neonatal forms. J Biol Chem 261:185–189
20. Tsuruya Y, Ikeda U, Kawakami K, Nagano K, Kamitani T, Oguchi A, Ebata H, Shimada K, Medford RM 1991 Augmented Na,K-ATPase gene expression in spontaneously hypertensive rat hearts. Clin Exp Hypertens [A] 13:1213–1222
21. Akera T, Ng Y-C 1991 Digitalis sensitivity of the Na+, K+-ATPase, myocytes and the heart. Life Sci 48:97–106
22. Sweadner KJ, Mcgrail KM, Khaw B 1992 Discoordinate regulation of isoforms of Na,K-ATPase and myosin heavy chain in the hypothyroid postnatal rat heart and skeletal muscle. J Biol Chem 267:769–773
23. Henning S 1981 Postnatal development: coordination of feeding, digestion, and metabolism. Am J Physiol 241:G199–G214
24. Aperia A, Larsson L 1984 Induced development of proximal tubular Na-KATPase, basolateral cell membranes and fluid reabsorption. Acta Physiol Scand 121:133–141
25. Beato M 1989 Gene regulation by steroid hormones. Cell 56:335–344
26. Pathak BG, Pugh DG, Lingrel JB 1990 Characterization of the 5′-flanking region of the human and rat Na,K-ATPase $\alpha 3$ gene. Genomics 8:641–647
27. Yagawa Y, Kawakami K, Nagano K 1990 Cloning and analysis of the 5′-flanking region of rat Na^+/K^+-ATPase $\alpha 1$ subunit gene. Biochim Biophys Acta 1049:286–292
28. Kawakami K, Yagawa Y, Nagano K 1990 Regulation of Na^+/K^+-ATPases. I. Cloning and analysis of the 5′-flanking region of rat NKAA2 gene encoding the $\alpha 2$ subunit. Gene 91:267–270
29. Celsi G, Wang Z-M, Aperia A 1992 Glucocorticoids regulate mRNA for Na^+,K^+-ATPase subunits in an age- and tissue-dependent manner. Pediatr Res 31:40A(abstr)
30. Ardekani AM, Walker SJ, Donohue SJ, Stitzel RE, Connors JM, Vrana KE 1989 Adrenocorticotropin and corticosterone levels in preweanling spontaneously hypertensive rats. Life Sci 44:919–925
31. Melikian J, Ismail-Beigi F 1991 Thyroid hormone regulation of Na,K-ATPase subunit-mRNA expression in neonatal rat myocardium. J Membr Biol 119:171–177