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Sensitive Periods for Glucocorticoids' Regulation of Na^+,K^+-ATPase mRNA in the Developing Lung and Kidney

GIANNI CELSI, ZHENG-MING WANG, GÖRAN AKUSJÄRVI, AND ANITA APERIA

Department of Pediatrics, St. Göran's Children's Hospital [G.C., Z.-M.W., A.A.] and Department of Microbial Genetics, Karolinska Institutet [G.A.], Stockholm, Sweden

ABSTRACT. We have previously reported that in the infant rat renal cortex, a saturating dose of glucocorticoid hormones (GC) rapidly increases the abundance of Na^+,K^+-ATPase mRNA. We now show that this effect is dose dependent. In the renal cortex of 10-d-old rats, an increase in renal Na^+,K^+-ATPase mRNA occurs with 2.5 μg betamethasone/100 g body weight. In subsequent experiments, performed 6 h after a saturating dose (60 μg/100 g body weight), we show that the effect is age dependent. The most marked effects on renal cortical α-mRNA were found at 10 d of age (5.3- ± 0.9-fold). A significant increase was also found in 20-d-old rats (1.6- ± 0.2-fold), but no effect was found in fetal and 5-d-old rats. Studies were also performed on the lung, where the most marked effect was noted in the perinatal period (2.0- ± 0.1-fold 2 d before birth and 1.76 ± 0.2 at 5 d of age), but no effect on α-mRNA was found at 10 and 20 d. In one protocol, the effect of betamethasone on renal Na^+K^+-ATPase mRNAs abundance was determined in adult adrenalectomized rats. In these rats, betamethasone induced a significant 1.6- ± 0.2-fold and 1.8- ± 0.3-fold increase in renal Na^+,K^+-ATPase mRNA. This effect, however, was significantly smaller than the increase induced in intact 10-d-old rats. GC induction of Na^+,K^+-ATPase mRNA is age and tissue dependent and is dependent on factors other than GC-receptor availability. The GC-sensitive period appears to coincide with the physiologic need for organ maturation. (*Pediatr Res* 33: 5–9, 1993)

Abbreviations

GC, glucocorticoid hormone
SSPE, sodium chloride, sodium phosphate, EDTA
ANOVA, analysis of variance

The adaptation of newborn mammals to extrauterine life is conditioned by an increased expression of enzymes that are of importance for specialized tissue function. Several studies suggest that circulating hormones modulate and coordinate the functional development of different organs by inducing enzyme maturation (1, 2). The GC may be the most important of the inductive hormones (2–6). In the rat, the circulating levels of corticosterone, the principal GC in this species, increase around the time of birth and then a second time before weaning (7–9). We recently reported that GC increase mRNA for both subunits of Na^+K^+-ATPase in renal tissue from preweanling rats (10). The effect was so rapid (~20 min) that it is likely to be a direct effect of the hormone on the transcription of Na^+K^+-ATPase subunit genes.

We have now examined whether the GC effect is developmentally regulated and, if so, whether the timing of the effect is tissue specific. Na^+K^+-ATPase is expressed in all eukaryotic cells and is of vital importance for the function of most tissues. We used tissue from lung and kidney, because these organs must rapidly mature in the perinatal and the weaning periods, respectively. To evaluate the role of GC receptor availability, the effect of a single dose of betamethasone on renal mRNA was also studied in adult adrenalectomized rats.

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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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MATERIALS AND METHODS

Animals. Experiments were performed on Sprague-Dawley rats (ALAB, Sollentuna, Sweden). Timed pregnant rats were housed individually and given free access to food and water. On gestational d 18 and 20 (*i.e.* 4 and 2 d before expected parturition), the rats received an intraperitoneal injection of betamethasone (Glaxo Labs, Ltd., Greenford, England) (60 μg/100 g body weight) or vehicle. Six h after the injection, betamethasone- and vehicle-treated rats were anesthetized with thiobutabarbital (8 mg/100 g body weight). Thereafter, fetuses were extracted within 10–15 min, and the fetal lungs and kidneys were removed. Renal cortex was rapidly dissected on ice. We pooled the lungs and renal cortices from three to five fetuses before homogenization. Infant rats were kept with their dams and were given an intraperitoneal injection of betamethasone (60 μg/100 g body weight) or vehicle at 5, 10, and 20 d of age. Six h after injection, they were anesthetized, lungs and kidneys were removed, and renal cortex was dissected on ice. In one protocol, 10-d-old rats were treated with various doses of betamethasone and the renal cortices were studied 6 h after injection.

In a separate protocol, bilateral surgical ablation of adrenals was performed under light ether anesthesia in 50-d-old rats. After surgery, the adrenalectomized animals received saline (0.9% NaCl) as drinking water. Control animals were sham-operated and thereafter given tap water. Five d after surgery, they were treated with betamethasone (60 μg/100 g body weight) 6 h before anesthesia with thiobutabarbital (8 mg/100 g body weight), the kidneys were immediately removed, and the cortex was dissected on ice.

Northern and dot blots. Total cellular RNA was isolated from pulmonary or renal cortical tissue with the Chirgwin method, and the integrity of the RNA was evaluated by electrophoresis in agarose/formaldehyde gels, as previously described (10). A representative Northern blot is shown in Figure 1. To quantify the mRNA levels, 4 μg (kidney) or 8 μg (lung) of total RNA were denatured in ice-cold 10 mM NaOH and blotted under vacuum onto a Hybond$^+$ nylon filter (Amersham, Buckinghamshire, UK)

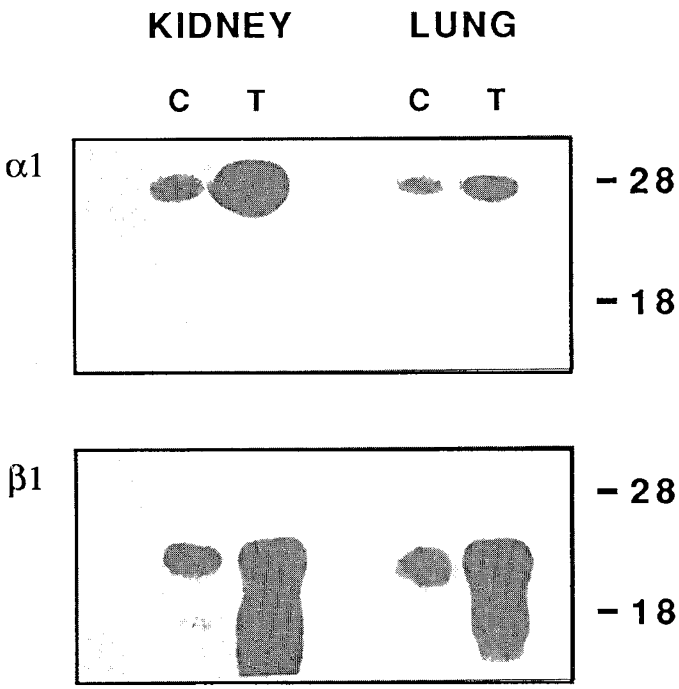


Fig. 1. Representative Northern blot analyses of renal cortex (from 10-d-old rats) and lung (from fetal rats). Rats were given injections of diluent (*C*) or betamethasone (*T*) (60 μg/100 g body weight) 6 h before the experiment. A total of 10 μg (kidney) or 20 μg (lung) of total RNA was loaded in each lane and probed with α1 and β1 cDNA. Positions of ribosomal 18S and 28S are indicated.

with a Bio-Blot SF unit (BioRad, Richmond, CA). Prehybridization (20 min) and hybridization (3 h) were performed at 65°C in Amersham Rapid Hybridization buffer. Approximately 10^6 dpm of [^{32}P]cDNA were added for the hybridization. The cDNA probes were random-primed (Multiprime DNA labeling system, Amersham) 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 exposure of the filters to Hyperfilm (Amersham) with two intensifying screens at −80°C. Multiple exposure of autoradiograms were made to ensure that the 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. 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) (11, 12). Actin cDNA was purchased from Clontech Laboratories, Palo Alto, CA. Autoradiogram bands were quantified with a LKB Ultrascan XL laser densitometer interfaced to an IBM PC. Peaks were integrated using LKB software. In all cases, triplicate RNA samples from betamethasone-treated and age-matched control rats were analyzed simultaneously on the same nylon filter. Each autoradiogram was scanned three times, and the mean was calculated and normalized to a standard (mRNA extracted from brain homogenate).

Statistical analysis. In both treated and control rats, five to six animals were studied in each group. Data are expressed as mean ± SEM. For clarity of presentation, the results are given as a fold increase over control values; however, statistical differences were always determined from the primary data. The variance in control animals was always lower than 10%. Statistical differences between betamethasone-treated and age-matched control rats were determined with the unpaired *t* test. Two-way ANOVA with the interaction test was used to analyze the influence of age, treatment, and the interaction between age and treatment.

RESULTS

The dose-response relationship between betamethasone and Na^+K^+-ATPase mRNA was determined in the renal cortex of 10-d-old rats. Six h after a single injection, betamethasone increased Na^+K^+-ATPase mRNA and the effect was dose-dependent (Fig. 2). An increase in α1 mRNA occurred with a 2.5 μg/100 g body weight. A similar dose of dexamethasone, a synthetic GC of equivalent potency, is thought to provide a physiologic replacement in adrenalectomized animals (13). This is also the threshold dose for stimulation of lung surfactant production in near-term rats (14) and for cardiac Na^+K^+-ATPase mRNA in 10-d-old rats (15). A maximal response was reached at approximately 60 μg/100 g body weight. A dose as high as 2500 μg/100 g body weight did not induce any further increase (data not shown). Similar responses were observed for the β1 mRNA. The following experiments were performed with 60 μg/100 g body weight. This dose has been shown to have a maximal inductive effect on renal cortical enzymes taken from infant rats (6) and on cardiac Na^+K^+-ATPase mRNA (15). This dose was therefore selected to ensure that the tissues were saturated with GC and to ensure a maximal effect also in the fetus.

The effect of betamethasone on renal Na^+K^+-ATPase gene expression in fetal and infant rats is shown in Figure 3. The age-dependent effect of betamethasone treatment on the expression of renal α1 and β1 mRNA was analyzed by a two-way ANOVA test. Both subunits were significantly affected by treatment ($p < 0.002$). Furthermore, both isoforms were affected differently by betamethasone at different ages ($p < 0.001$ for both). In the fetus, at 2 d before parturition, betamethasone did not significantly change α1 and β1 mRNA abundance. In 5-d-old rats, α1 mRNA abundance was not significantly altered after betamethasone, but β1 mRNA abundance was increased 2-fold ($p < 0.05$). In 10-d-old rats, betamethasone increased both α1 and β1 mRNA 5-fold ($p < 0.001$). In 20-d-old rats, α1 and β1 mRNA were also significantly increased after betamethasone treatment (1.6- and 2.5-fold, $p < 0.05$ and 0.01, respectively) and the effect was less pronounced than in 10-d-old rats.

Similar results were obtained if Na^+K^+-ATPase α1 and β1 were related to actin mRNA, because actin mRNA abundance was not altered by betamethasone in any of the age groups (data not shown).

In one protocol, the effect of betamethasone on renal Na^+K^+-ATPase mRNA abundance was determined in adult adrenalec-

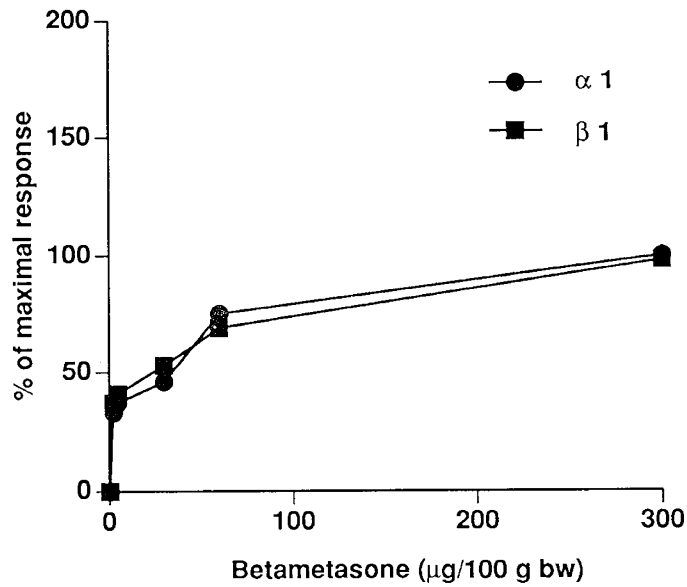
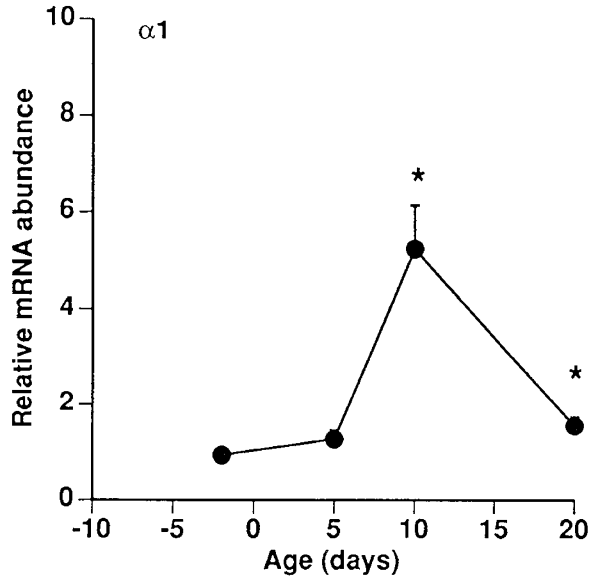


Fig. 2. Effect of different doses of betamethasone on Na^+,K^+-ATPase α1 isoforms of mRNA abundance in renal cortex from 10-d-old rats. Rats were studied 6 h after injection.



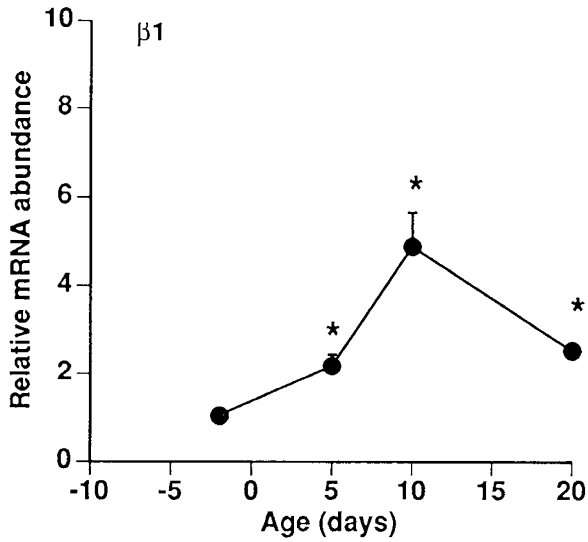


Fig. 3. Effect of betamethasone treatment on Na^+,K^+-ATPase $\alpha 1$ and $\beta 1$ isoforms of mRNA abundance in renal cortex from rats of different ages. Rats were given injections of betamethasone (60 μg/100 g body weight) 6 h before experiment. An arbitrary value of 1 has been given to mRNA abundance in control rats. *Star* indicates significant difference as compared with control rats.

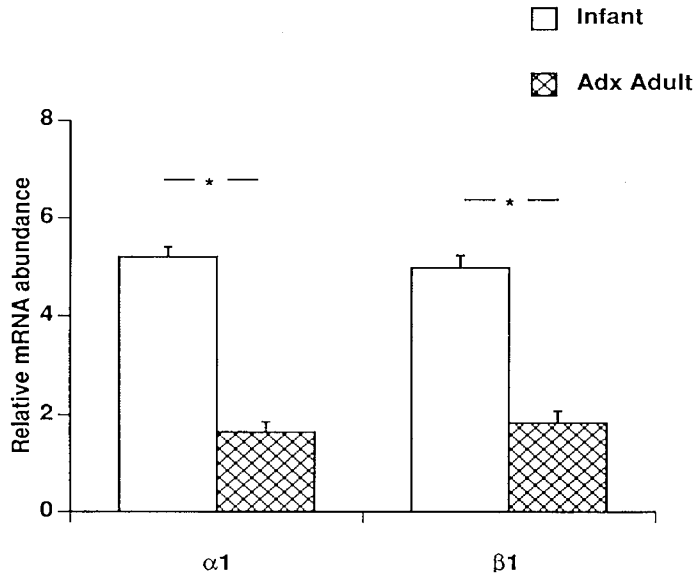


Fig. 4. Effect of betamethasone treatment on Na^+,K^+-ATPase $\alpha 1$ and $\beta 1$ isoforms of mRNA abundance in the renal cortex from normal infant (10-d-old) and from adult adrenalectomized rats. Rats were given injections of betamethasone (60 μg/100 g body weight) 6 h before the experiment. An arbitrary value of 1 has been given to mRNA abundance in control rats. Values are means ± SEM. *Star* indicates significant difference as compared with control rats.

tomized rats. When the receptor availability was maximally up-regulated, *i.e.* 5 d after adrenalectomy (16), a single injection of betamethasone in the adult adrenalectomized rats induced a borderline, but significant, increase in $\alpha 1$ and $\beta 1$ mRNA in the renal cortex (1.6- ± 0.2-fold and 1.8- ± 0.3-fold, respectively, $p < 0.05$ for both) (Fig. 4). This effect, however, was significantly smaller than the increase induced in intact 10-d-old rats ($p < 0.001$).

The age-dependent effect of betamethasone on pulmonary mRNA is shown in Figure 5. Both subunits were significantly affected by treatment ($p < 0.001$, two-way ANOVA test). Furthermore, both isoforms were affected differently by betamethasone at different ages ($p < 0.001$ for both, two-way ANOVA test). Betamethasone caused a significant increase in $\alpha 1$ and $\beta 1$ mRNA abundance in the fetal lung. In treated rats, the abundance of both $\alpha 1$ and $\beta 1$ mRNA was 1.5-fold higher than in control rats ($p < 0.05$ in both) 4 d before parturition and 2-fold ($p < 0.001$) 2 d before parturition. In 5-d-old rats, betamethasone had a borderline effect on $\alpha 1$ mRNA (1.7-fold increase, $p = 0.05$), but the mRNA for $\beta 1$ was 3.6-fold increased ($p < 0.001$) after treatment. In 10- and 20-d-old rats, betamethasone did not change $\alpha 1$ mRNA abundance, but it increased the $\beta 1$ mRNA content 3- and 2-fold ($p < 0.001$ in both). The Na^+K^+-ATPase mRNA level was not related to actin mRNA, because betamethasone significantly reduced the pulmonary actin mRNA abundance in 20-d-old rats (50% of control, $p < 0.001$). Actin mRNA was not significantly changed after betamethasone in younger animals (data not shown).

Because low levels of $\alpha 2$ and $\alpha 3$ mRNA have been reported as present in the lung (17), we also studied whether betamethasone might induce the expression of these isoforms. Very low levels of $\alpha 2$ mRNA were detected in all age groups, but no effect of GC was observed. Because of the very low expression of this isoform, no attempt at densitometric quantitation has been made. The mRNA for $\alpha 3$ isoform was not detected in any age group.

DISCUSSION

This study shows that GC can up-regulate the mRNA for Na^+,K^+-ATPase in the immature lung as well as in preweaning kidney. The inductive effect of GC was observed only during a limited period. The maximal sensitivity of both $\alpha 1$ and $\beta 1$ mRNA to GC was found in the lung in the perinatal period and in the kidney in the preweaning period.

Can the age- and tissue-dependent response to GC be due mainly to differences in GC-receptor availability? The GC hormone-receptor complex seems to be present in both the lung and kidney throughout late fetal life and infancy (18–20). The lack of response in fetal kidney and in preweaning lung cannot therefore be explained by absence of receptors. The results of the study in adult adrenalectomized animals also suggest that receptor availability is not a rate-limiting step for GC response. After adrenalectomy, the number of GC receptors is maximally up-regulated in adult rats, so that their concentration is equal or even higher than in infant rats (16). Yet betamethasone did not elicit the same increase in renal mRNA abundance in adult adrenalectomized rats as it did in 10-d-old rats.

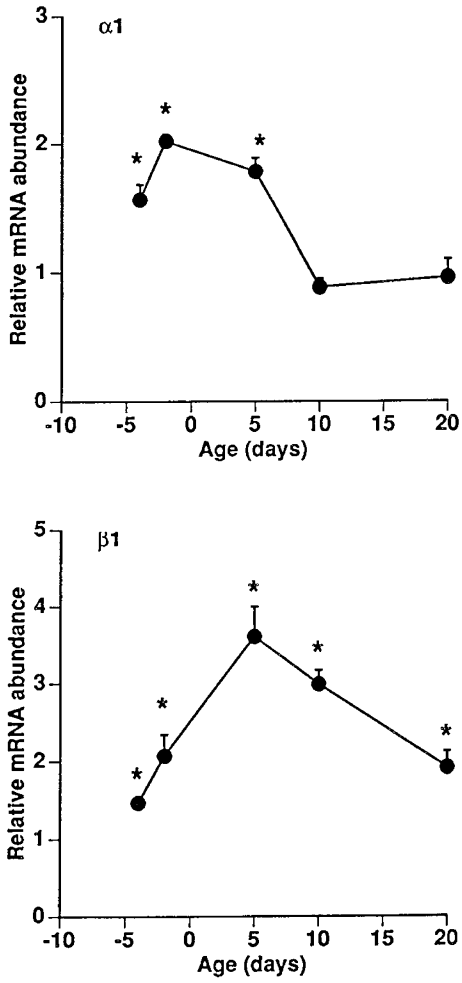


Fig. 5. Effect of betamethasone treatment on Na^+,K^+-ATPase $\alpha 1$ and $\beta 1$ isoforms mRNA abundance in lung from rats of different ages. Rats were given injections of betamethasone (60 μg/100 g body weight) 6 h before the experiment. An arbitrary value of 1 has been given to mRNA abundance in control rats. Values are means ± SEM. *Star* indicates significant difference as compared with control rats.

A more important explanation for the developmental differences in GC sensitivity may be the fact that the GC hormone receptor complex interacts with other transcriptional factors to regulate Na^+,K^+-ATPase mRNA and that the expression of these factors is tissue and age dependent. Binding domains for several transcriptional factors, such as AP-1, Sp1, and CREB, have been identified in the promoter region of the Na^+,K^+-ATPase gene, in addition to GC-binding domains (21). The GC effect might be dependent on the expression of these factors. Thus, the interaction between the GC hormone-receptor complex and these factors may vary between mutual repression and activation depending on the factor that is in excess (22). An important topic for future research will be to study the expression of these transcriptional factors and the regulation of their binding to the promoter region of Na^+,K^+-ATPase genes in developing pulmonary and renal tissues.

In the kidney, the $\alpha 1$ and $\beta 1$ mRNA abundance always changed in parallel after GC treatment. In the lung, however, a disproportionate increase in $\beta 1$ mRNA occurred in 10- and 20-d-old rats. A similar disproportionate increase in $\beta 1$ over $\alpha 1$ mRNA has been reported in the rat heart after thyroid hormone treatment and in LLC-PK1 cell incubated in a low K^+ media (23, 24). The significance of this selective increase in β mRNA has not been established.

Na^+,K^+-ATPase is an important determinant of cell electrolyte homeostasis and cell volume. It can also modulate tissue-specific functions, such as salt and water reabsorption in the renal tubule. Several lines of evidence suggest that the low availability of Na^+,K^+-ATPase in renal tissue (25) limits both the capacity to retain sodium (26) and the capacity to concentrate the urine (27). In the lung, Na^+,K^+-ATPase provides the energy for the vectorial transport of fluid and electrolytes from the lumen to the perialveolar space across the alveolar membrane. In alveolar type II cells, which are the most important cells for fluid reabsorption, the ouabain-sensitive uptake of $^{86}Rb^+$, an index of Na^+,K^+-ATPase-mediated ion transport, increases 3-fold postnatally (28). The present results suggest that the physiologic upsurge of GC that occurs at birth (7, 8) might help to improve pulmonary function in the neonate by stimulating the maturation of Na^+,K^+-ATPase, which in turn would improve fluid drainage from the alveoli. It is noteworthy that the perinatal hormonal upsurge of circulating GC coincides with both an increase in Na^+,K^+-ATPase mRNA abundance (17) and activity (28) and with an increase in transepithelial fluid transport in the lung (29), whereas the preweaning GC upsurge coincides with a similar cascade of events in the kidney (27). The results of the present study suggest that terminal differentiation in mammals is determined by the interaction between central endocrine systems and developmentally regulated transcriptional factors in each organ system.

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