Abstract. Transepithelial Na⁺ reabsorption across tight epithelia is regulated by aldosterone. Mineralocorticoids modulate the expression of a number of proteins. Na⁺,K⁺-ATPase has been identified as an aldosterone-induced protein (Geering, K., M. Girardet, C. Bron, J. P. Kraehenbuhl, and B. C. Rossier, 1982, J. Biol. Chem., 257:10338-10343). Using A6 cells (kidney of Xenopus laevis) grown on filters we demonstrated by Northern blot analysis that the induction of Na⁺,K⁺-ATPase was mainly mediated by a two- to fourfold accumulation of both α- and β-subunit mRNAs. The specific competitor spironolactone decreased basal Na⁺ transport, Na⁺,K⁺-ATPase mRNA, and the relative rate of protein biosynthesis, and it blocked the response to aldosterone. Cycloheximide inhibited the aldosterone-dependent sodium transport but did not significantly affect the cytoplasmic accumulation of Na⁺,K⁺-ATPase mRNA induced by aldosterone.
ture conditions on plastic petri dishes and collagen-coated filters are described elsewhere (10, 22, 37). For this study, nitrocellulose (0.45 μm; Millipore Continental Water Systems, Bedford, MA) or polycarbonate (6 μm; Nucleopore, Pleasanton, CA) filters were used. Culture media were supplemented with 5% FCS from Gibco (Basel, Switzerland). Two seeding densities were used for filter cultures: 1.5 × 10^6 cells/cm² or 0.3 × 10^6 cells/cm². Cells were kept for 15 d in culture before the experiment. All experiments on filter-grown cells were performed with a cloned cell line (A6-2F3) obtained by limit-dilution and selected for its high Na⁺ transport, its high transmural resistance, and its responsiveness to aldosterone. Aldosterone was a gift from Ciba-Geigy (Basel, Switzerland); spironolactone was a gift from Searle (San Jose, Puerto Rico); and cycloheximide was from Sigma Chemical Co. (St. Louis, MO). Measurement of potential difference (PD) and short circuit current (SCC) were performed as described (22, 37).

**Na⁺,K⁺-ATPase Protein Biosynthesis**

Cells were labeled with 200 μCi/ml [35S]methionine (Amersham International, Amersham, UK) for 45 min in serum-free medium. Cells were solubilized according to Rose et al. (26). TCA precipitates were counted and tested for protein content (16). Equal amounts of counts and protein were submitted to immunoprecipitation (7) with rabbit antiserum (anti-α-subunit from *Bufo marinus*) (9), and anti-β-subunit from *X. laevis* (8). If required, the protein concentration of the immunoprecipitates was normalized by addition of unlabeled A6 homogenates. SDS PAGE and fluorography were performed as described elsewhere (7). Quantification of the bands was performed at 515 nm with a Zeiss densitometric scanning device.

**Northern Blot Analysis of Cytoplasmic RNA**

RNA was prepared by the citric acid method (8, 32). Equal amounts of denatured cytoplasmic RNA (1–2 μg) were run on 1% agarose/formaldehyde gels and transferred to nitrocellulose filters (17). Alternatively glyoxylated RNA was separated by electrophoresis in phosphate buffer (38). Hybridizations were performed as described (38) with an excess of nick-translated probes (specific activities: 0.3–2 × 10⁸ cpm/μg DNA) (17). α- and β-Subunits were revealed with pEX2a4a and pEX2β8a, respectively (Verrey, E, P. Kairouz, E. Schaerer, P. Zoerkler, M. P. Paccolat, K. Geering, B. C. Rossier, and J. P. Kraehenbuhl, manuscript in preparation). These clones have been identified by immunoscreening of plasmid expression libraries prepared from size-fractionated poly A⁺ RNA of A6 cells. pEX2a4a and pEX2β8a contains a 1.4-kb and a 0.55-kb insert, respectively. Identity of both clones has been confirmed by retroblotting, hybrid-selected translation, and sequence comparison to published sequences (13–15, 21, 35, 36). Cytoskeletal actin-specific RNAs were monitored with pAL41 (1). Densitometric scanning was performed as described above.

**Results**

To analyze the effect of aldosterone, cells were incubated from 6 to 96 h with the hormone. Sodium transport was monitored and cells were collected for measurement of mRNA levels and the rate of protein synthesis of each subunit of Na⁺,K⁺-ATPase as described in Materials and Methods.

As shown in Fig. 1 and Table I, aldosterone treatment resulted in a two- to fourfold accumulation of cytoplasmic α- and β-specific mRNA within a 6-h period. No further increase was observed between 6 and 96 h of stimulation. By Northern blot analysis (Fig. 1 a), the α- and β-subunit mRNA migrate as major bands with the size of 3.9 and 2.6 kb consistent with the results reported for other species (13–15, 33, 35, 36). The degree of hormonal induction was measured by comparing the signals obtained on the same gel with a β-actin mouse probe pAL41 (1). Cytoskeletal actin mRNAs were detected (Fig. 1 a, left panel) as two bands which represent at least three different cytoskeletal actins of A6 cells (20, 41). Aldosterone did not significantly modulate the cytoplasmic level of these actin mRNAs, in agreement with previous observations made at the protein level (39).

After 6 h of stimulation with aldosterone, the relative rate of α- and β-subunit polypeptide synthesis increased 1.65- and 2.0-fold (Table I), and a two- to threefold increase was measured after 96 h of stimulation. As expected (Fig. 1 b), the immunoprecipitation procedure revealed selectively the α-subunit (M₀, ~98,000) and the β-subunit as a 42-kD core-glycosylated and a 49-kD terminally glycosylated form (8, 22).

Finally, 6 h of aldosterone treatment resulted in a 3.5-fold increase of sodium transport but, and in contrast to the effect on mRNA and protein synthesis, a significant further increase in sodium transport (8.7-fold) was observed after 96 h of hormonal stimulation (Fig. 1 c; Table I).
Table I. Effect of Aldosterone (300 nM) and Spironolactone (300 μM) on A6 cells (2F3) Grown on Filters

| Fractional change: test/control | Spironolactone 6 h | Control 6 h | Aldosterone 6 h | Aldosterone 96 h |
|--------------------------------|--------------------|-------------|----------------|-----------------|
|                                | mean (range) or SE | n           | mean (range) or SE | n              |
| Cytoplasmic mRNA               |                    |             |                  |                 |
| Na⁺,K⁺-ATPase α-subunit        | 0.79 (0.43-1.08)    | 3           | 1.00             | 4 (1.71-3.62)   | 4 (2.51-2.71) | 2 |
| Na⁺,K⁺-ATPase β-subunit        | 0.56 (0.27-1.08)    | 3           | 1.00             | 3.82 (3.06-5.09)| 4 (2.61-3.41)| 2 |
| Actin                          | 0.81 (0.75-0.85)    | 3           | 1.00             | 1.11 (0.86-1.67)| 4 (0.92-1.57)| 2 |
| Rate of protein synthesis      |                    |             |                  |                 |
| Na⁺,K⁺-ATPase α-subunit        | 0.29 (0.15-0.53)    | 3           | 1.00             | 1.65 (1.52-1.84)| 3 (2.65-2.90)| 2 |
| Na⁺,K⁺-ATPase β-subunit        | 0.32 (0.10-0.61)    | 3           | 1.00             | 2.03 (1.40-2.42)| 3 (1.30-2.73)| 2 |
| Sodium transport               |                    |             |                  |                 |
| Short-circuit current (SCC)    | 0.39 ± 0.05         | 11          | 1.00             | 3.52 ± 0.33     | 12 ± 1.28 | 7 |
| Potential difference (PD)      | 0.64 ± 0.10         | 11          | 1.00             | 2.88 ± 0.34     | 12 ± 0.71 | 7 |
| Resistance (R)                 | 1.76 ± 0.43         | 10          | 1.00             | 0.87 ± 0.08     | 11 ± 0.12 | 7 |

Mean of 2-4 independent experiments expressed as fractional change: test/control (range is indicated in parenthesis). Control is arbitrarily set as 1.0 and represents dishes incubated in presence of 5% FCS and diluent (1% ethanol). SE, standard error. PD, of controls was 19.4 ± 3.0 (SE) mV; SCCo (controls) = 1.8 μA/cm² ± 0.3 (SE); R, (control) = 11,143 ± 850 (SE) ohm cm².

Since our control cells are incubated in the presence of 5% FCS which might contain significant amounts of adrenal steroid hormone and/or unknown factors modulating the mineralocorticoid receptor, we tested the effect of spironolactone, a specific competitive antagonist for the binding to the mineralocorticoid type 1 and type 2 receptor (2). Indeed, spironolactone (with antagonist/agonist molar ratio of 1,000) was able to inhibit 90-95% of the aldosterone-induced sodium transport, mRNA, and protein responses (data not shown). At the same concentration, spironolactone per se elicited a slight decrease in all parameters tested (Table I) suggesting that our "control" dishes are somewhat stimulated by mineralocorticoid hormones (and/or factors). This is further substantiated by the fact that spironolactone per se increased transmural resistance (PD/SCC) by 76% while aldosterone decreased transmural resistance by 13-33% as reported previously in the toad bladder (6) and A6 cells (42). Since it was not possible to maintain A6 cell in serum-free media for an extended period of time, it would appear that spironolactone is a useful drug to set a mineralocorticoid “free” base line. Similarly tamoxiphen (an estrogen antagonist) has been used to set an estrogen-free baseline for studying the estrogen-dependent induction of vitellogenin (34).

In the course of the present study and in a previous study (22), we observed that a number of factors can influence the sodium transport response. These factors include the type of growth medium (FCS), the density of seeding, the frequency of medium changes. By contrast, these factors do not appear to modify significantly the effect of aldosterone on mRNA level or on protein synthesis. These latter responses might thus be part of a primary steroid response triggered independently of transepithelial sodium transport. This hypothesis was tested on A6 cells grown on plastic which are less differentiated in two important aspects: (a) they do not express a detectable amiloride-sensitive sodium uptake at their apical border (31); (b) the total immunochemical pool of Na⁺,K⁺-ATPase is three- to sixfold lower compared to that of cells grown on collagen-coated filters (22).

As shown in Fig. 2 and Table II, aldosterone elicited after 6 h a parallel increase (1.8-2.3-fold) in mRNA accumulation and protein synthesis rate. This induction is comparable but somewhat smaller to that described for A6 cells grown on collagen-coated filter cups (Fig. 1, Table I). This effect is however transient as mRNA levels return to unstimulated level within 96 h. Similar transient effects were previously observed at the protein level (22). The sustained response observed with cells grown on filter cups might be dependent on the state of differentiation and/or transepithelial sodium transport per se.

![Figure 2. Northern blot of Na⁺,K⁺-ATPase from A6 cells grown on plastic dishes (see legend to Fig. 1 for symbols).](image-url)
Table II. Effect of Aldosterone (300 nM) on A6 Cells Grown on Plastic

| Cytoplasmic mRNA                                    | Control mean | Aldo 6 h mean (range) | Aldo 24-96 h mean (range) |
|----------------------------------------------------|--------------|-----------------------|---------------------------|
| Na⁺,K⁺-ATPase α-subunit                            | 1.0          | 1.76 (1.37-2.00)      | 0.91 (0.72-1.10)          |
| Na⁺,K⁺-ATPase β-subunit                            | 1.0          | 2.19 (1.77-2.77)      | 1.22 (1.10-1.34)          |
| Actin                                              | 1.0          | 1.24 (1.08-1.34)      | 1.02 (1.02-1.02)          |

Rate of protein synthesis

| Na⁺,K⁺-ATPase α-subunit                            | 1.0          | 2.06 (1.75-2.63)      |                           |
| Na⁺,K⁺-ATPase β-subunit                            | 1.0          | 2.34 (1.75-2.73)      |                           |

Mean of two or three independent experiments expressed as fractional change (test/control). Control is arbitrarily set as 1.0 and represents dishes incubated in presence of 5% FCS and diluent (1% ethanol).

Effects of Cycloheximide

Cycloheximide is a translation inhibitor which has been shown to inhibit the aldosterone-dependent sodium transport in the amphibian urinary bladder (4). In A6 cells grown on filters, we first tested increasing concentrations of the drug on total protein synthesis and electrophysiological parameters of sodium transport during a 6-h incubation. To measure total protein synthesis, incorporation of [35S]methionine into proteins was monitored during the first and the last hours of the incubation with cycloheximide. At 20 μg/ml cycloheximide, more than 95% of the protein synthesis was inhibited as expected but a dramatic fall in transmural electrical resistance was also observed, suggesting the disruption of epithelial polarity. At 1.5 μg/ml cycloheximide, ~90% of the protein synthesis was inhibited and transmural electrical resistance fell progressively during the last 4 h of incubation (data not shown). At 0.5 μg/ml cycloheximide, ~75% of protein synthesis was inhibited without fall of transmural resistance. As shown in Table III, this degree of inhibition was, however, sufficient to block the aldosterone-dependent increase of sodium transport by >90%. This concentration was therefore selected for the experiment shown below.

As shown in Fig. 3 and Table III, cycloheximide per se did not change the cytoplasmic level of α, β, and actin mRNAs for up to 6 h of incubation. Aldosterone alone elicited a 2.4-4.0-fold increase of α- and β-subunit mRNA. Cycloheximide did not prevent significantly the aldosterone-dependent induction of α mRNA (1.8-fold) or that of β mRNA (4.6-fold).

At a low dose (0.5 μg/ml), cycloheximide did not change actin mRNA but at high dose (≥1.5 μg/ml) a significant increase was observed (approximately twofold) as previously reported (3, 23) (data not shown).

Discussion

Hormonal induction of Na⁺ reabsorption across tight epithelia by aldosterone involves permeability changes at the apical and basolateral membrane. The early increase of reabsorption is thought to be due to an opening of Na⁺ channels at the apical membrane (31). The proteins involved in the mediation of this early response have not yet been identified. A later increase in Na⁺ transport is accompanied by an increase of Na⁺,K⁺-ATPase protein biosynthesis (6, 7, 28). In this study, we analyze the hormone induction of this enzyme on the molecular level in the A6 cell line derived from X. laevis kidney. This experimental system combines the advantages of an established cloned cell line with the maintenance of a high degree of differentiation and allows us to follow the biogenesis of Na⁺,K⁺-ATPase both at the nucleic acid and protein level in the same cells in which the full sodium transport response to aldosterone can be monitored. In such a system, the steroid hormone response might ultimately be understood both in molecular and physiological terms. In the present study, we have focused on the possible role of Na⁺,K⁺-ATPase induction in the expression of aldosterone-dependent sodium transport. We would like to discuss the following three points.

The Role of Protein Synthesis in the Induction of α- and β-Subunit mRNAs by Aldosterone

The role of protein synthesis during steroid induction has been investigated in a number of different experimental systems. For example, the induction of mouse mammary tumor virus by glucocorticoid occurs as a result of a 10-fold ac-
cumulation of mouse mammary tumor virus RNA, an effect which is not blocked by protein synthesis inhibitors (24, 25), suggesting a direct transcriptional control by the glucocorticoid receptor complex. More recent experiments have shown that dexamethasone increases the number of RNA polymerase II molecules transcribing mouse mammary tumor virus DNA (5). In other cases, the situation appears to be quite different. Thus, the induction of ovalbumin and conalbumin mRNA by estrogen and progesterone measured in chick oviduct explant cultures is fully blocked by cycloheximide or puromycin (18) under conditions leading to only 70-80% inhibition of total protein synthesis. In this case, the induction of ovalbumin and conalbumin which is not blocked by protein synthesis inhibitors (24, 25), suggesting a direct transcriptional control by the glucocorticoid hormones or drugs (6, 7, 28, 40). This is a rather striking observation since each subunit is coded for by distinct species of mRNAs (2.6 kb vs. 3.9 kb) which can be translated in a cell-free system and inserted independently into endoplasmic reticulum (8). In the present study, we also observe a coordinate effect of aldosterone on the rate of synthesis of each subunit in three different experimental conditions (Tables I-III). In addition, the data obtained at the protein level are more consistent with the concept that Na⁺,K⁺-ATPase mRNA accumulation is the result of a direct effect of the aldosterone-receptor complex on Na⁺,K⁺-ATPase expression and occurs without major change in cell protein(s), or, alternatively, in presence of a residual 25% protein synthesis. Interestingly, in the X. laevis liver it has been shown that cycloheximide in vivo (leading to >95% inhibition of total protein synthesis) did not block the activation of vitellogenin gene transcription in response to estrogen (II). We may be dealing with a rather similar situation in the A6 model although the two experimental systems differ in one respect: the vitellogenin gene is activated from a practically silent state while Na⁺,K⁺-ATPase belongs to the category of housekeeping genes with a significant basal level of transcriptional activity. Our data do not provide direct evidence for activation of gene transcription by aldosterone. Nuclear run on experiments will provide us with further and more direct insight into this question. One should, however, point out that a direct effect on gene transcription is not mutually exclusive with other mechanisms operating at the posttranscriptional level (mRNA processing, transport, and stability), the translational level, or at the posttranslational level. The cDNAs and antibodies available should help us to distinguish in the future between these possibilities. 

### Table III. Effect of Cycloheximide (0.5 μg/ml) on Aldosterone-dependent Induction of Na⁺,K⁺-ATPase and Sodium Transport

| Cytoplasmic mRNA | Fractional change: test/control |
|------------------|---------------------------------|
| **Na⁺,K⁺-ATPase α-subunit** | + Cycloheximide 6 h |
| Amount (mean (range)) | n | + Aldosterone 6 h | + Aldosterone 6 h |
| Na⁺,K⁺-ATPase α-subunit | 1.0 | 3 | 2.42 | 0.98 | 1.76 |
| | 3 | (1.71-3.62) | 3 | (0.78-1.15) | (1.36-2.19) | 3 |
| Na⁺,K⁺-ATPase β-subunit | 1.0 | 3 | 4.06 | 1.22 | 4.65 |
| | 3 | (3.06-5.09) | 3 | (0.77-1.47) | (3.69-6.25) | 3 |
| Actin | 1.0 | 3 | 1.19 | 1.04 | 1.29 |
| | 3 | (0.87-1.67) | 3 | (0.93-1.21) | (1.29-1.33) | 3 |
| Sodium transport | 1.0 | 5 | 2.86 | 0.57 | 0.70 |
| Short-circuit current (SCC) | 1.0 | 5 | ± 0.25 (SE) | ± 0.08 (SE) | ± 0.18 (SE) |
| Potential difference (PD) | 1.0 | 5 | 2.76 | 0.75 | 1.01 |
| | 5 | ± 0.45 (SE) | 5 | ± 0.15 (SE) | ± 0.20 (SE) |
| Resistance (R) | 1.0 | 5 | 0.98 | 1.40 | 1.76 |
| | 5 | ± 0.14 (SE) | 5 | ± 0.29 (SE) | ± 0.46 (SE) |

Mean of three independent experiments expressed as fractional change (test/control). Control is arbitrarily set as 1.0 and represents dishes incubated in absence of aldosterone and cycloheximide, in presence of 5% FCS and diluent. PDo (control) = 19.6 ± 3.6 (SE) mV; SCCo = 2.2 ± 0.3 μA/cm²; Ro = 9161 ± 1876 ohm-cm².

In the present study, aldosterone-dependent sodium transport was found to be particularly sensitive to the protein synthesis inhibitor. Indeed 75% inhibition of total protein synthesis (0.5 μg/ml cycloheximide) abolished >90% of the physiological response (Table III), a result very similar to that observed in the toad urinary bladder (4). In the absence of a significant effect on sodium transport, aldosterone was still able to promote the accumulation of β mRNA and α mRNA (Fig. 3, Table III). Our data do not favor the idea of an aldosterone-dependent induction of an intermediate (regulatory) protein that in turn controls the Na⁺,K⁺-ATPase gene transcription or posttranscriptional events. Our data are more consistent with the concept that Na⁺,K⁺-ATPase mRNA accumulation is the result of a direct effect of the aldosterone-receptor complex on Na⁺,K⁺-ATPase expression and occurs without major change in cell protein(s), or, alternatively, in presence of a residual 25% protein synthesis. Interestingly, in the X. laevis liver it has been shown that cycloheximide in vivo (leading to >95% inhibition of total protein synthesis) did not block the activation of vitellogenin gene transcription in response to estrogen (II). We may be dealing with a rather similar situation in the A6 model although the two experimental systems differ in one respect: the vitellogenin gene is activated from a practically silent state while Na⁺,K⁺-ATPase belongs to the category of housekeeping genes with a significant basal level of transcriptional activity. Our data do not provide direct evidence for activation of gene transcription by aldosterone. Nuclear run on experiments will provide us with further and more direct insight into this question. One should, however, point out that a direct effect on gene transcription is not mutually exclusive with other mechanisms operating at the posttranscriptional level (mRNA processing, transport, and stability), the translational level, or at the posttranslational level. The cDNAs and antibodies available should help us to distinguish in the future between these possibilities.

### The Coordinate Control of α and β mRNA Accumulation and the Rate of Enzyme Subunit Synthesis by Aldosterone

We have previously shown that the synthesis of both α- and β-subunit of Na⁺,K⁺-ATPase is remarkably coordinated. A parallel change in the rate of synthesis of each subunit is observed whether the system is up- or down-regulated by hormones or drugs (6, 7, 28, 40). This is a rather striking observation since each subunit is coded for by distinct species of mRNAs (2.6 kb vs. 3.9 kb) which can be translated in a cell-free system and inserted independently into endoplasmic reticulum (8). In the present study, we also observe a coordinate effect of aldosterone on the rate of synthesis of each subunit in three different experimental conditions (Tables I-III). In addition, the data obtained at the protein level are paralleled by a coordinate accumulation or disappearance of the corresponding cytoplasmic mRNAs. Within each experimental condition (Figs. 1, 2, and 3), there was no significant difference of induction between the mRNA coding for α- and β-subunit, respectively. However, a distinct
trend toward a larger accumulation of β mRNA was observed after 6 h of induction by aldosterone, especially in the presence of cycloheximide (0.5 μg/ml). At present we have no rational explanation for this discrepancy.

**Relationship between the Induction of Na⁺,K⁺-ATPase by Aldosterone and the Sodium Transport Response**

Our data indicate that the increase in the relative rate of synthesis of Na⁺,K⁺-ATPase subunits can largely be attributed to a corresponding accumulation of specific RNA. This induction appears to be mineralocorticoid specific since it is antagonized by spironolactone. Indeed, a good correlation between the mRNA level, the rate of subunit synthesis, and the sodium transport is observed during aldosterone induction and spironolactone inhibition (Table I). Such a relationship can be predicted if one postulates that the translation of α and β mRNA is not under aldosterone control. Our observation that spironolactone consistently decreased the rate of subunit synthesis more than it diminished mRNA content suggests that this prediction might not be correct in all circumstances. The interesting possibility that some translational control is induced by aldosterone (at probably low levels of receptor occupancy) and turned off by spironolactone should be considered.

During the first 6 h of induction (or inhibition in presence of spironolactone), a good correlation between the sodium transport response and Na⁺,K⁺-ATPase gene expression is observed. It is important to note that such a relationship was not necessarily anticipated since the sodium transport response depends on many factors modulating both apical and basolateral membrane components.

Our data support the concept that the induction of Na⁺,K⁺-ATPase is one, maybe necessary but not sufficient, of the many steps required to establish a full and integrated the regulation of apical determinants; namely, the amiloride-basolateral membrane components.

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