Early Effect of Aldosterone on the Rate of Synthesis of the Epithelial Sodium Channel α Subunit in A6 Renal Cells

ANNE MAY, ALESSANDRO PUOTI, HANS-PETER GAEGGELER, JEAN-DANIEL HORISBERGER, and BERNARD C. ROSSIER
Institut de Pharmacologie et de Toxicologie de l'Université, Lausanne, Switzerland.

Abstract. Transepithelial Na\textsuperscript{+} reabsorption across tight epithelia is regulated by aldosterone. The amiloride-sensitive epithelial sodium channel (ENaC) is a major target for the natriferic action of aldosterone. In this study, the effect of aldosterone on ENaC mRNA abundance and the rate of protein synthesis for each of the three ENaC subunits (α, β, and γ) in the A6 kidney cell line were examined. In cells grown on plastic, aldosterone induced a large and rapid increase in epithelial sodium channel (ENaC) β and γ subunit mRNA abundance, but this effect is not translated into the synthesis of the corresponding proteins. In cells grown on a porous substrate, amiloride-sensitive electrogenic sodium transport was expressed and was upregulated by aldosterone (300 nM) as early as 1 h after the addition of the hormone. The α, β, and γ mRNA abundance was not changed by aldosterone during the first 3 h of stimulation, whereas a fourfold increase over control was observed after 24 h. The rate of synthesis of α subunit was significantly increased above control already 60 min after aldosterone addition, whereas β subunit synthesis increased only 6 h after hormone addition, with no significant change for the γ subunit. The half-lives of each subunit as assessed by \textsuperscript{35}S methionine pulse-chase experiments were short (between 40 and 50 min) and were not modified by aldosterone. Taking into account the short half-life of ENaC protein and assuming that the synthesis of the α subunit is a limiting factor in the assembly and expression of new channels at the cell surface, it is proposed that the aldosterone regulation of sodium transport might be, in part, mediated by de novo synthesis of the channel protein. (J Am Soc Nephrol 8: 1813–1822, 1997)
presented here, we have examined the possible role of ENaC mRNA accumulation and/or protein synthesis in establishing an aldosterone-dependent, amiloride-sensitive electrogenic sodium transport. First, we have examined the regulation of ENaC by aldosterone in cells grown on plastic (i.e., which do not express an amiloride-sensitive pathway in the apical membrane) and in cells grown on filter cups (i.e., which do express an aldosterone-dependent amiloride-sensitive pathway).

In cells that do not express an amiloride-sensitive sodium transport, aldosterone induces a large change in mRNA abundance, coding for β and γ xENaC, but apparently this effect is not translated into a significant amount of the corresponding proteins, explaining a lack of detectable amiloride-sensitive sodium transport in these cells.

In contrast, we show that during the early phase of aldosterone action, aldosterone has a major effect on the translation of the α xENaC, which has been previously shown to be limiting in the assembly of active channels to the plasma membrane of Xenopus oocytes (10). Aldosterone induces accumulation of α, β, and γ transcripts only during the late phase of its action, which could lead to a further increase in channel protein synthesis.

**Materials and Methods**

**Cell Culture**

The 2F3 subclone of A6 cells (American Tissue Type Collection, Rockville, MD), obtained by limiting dilution and selected for its high Na⁺ transport, its high transmural resistance, and its responsiveness to aldosterone, was used for all experiments (passages 86 to 96) (11).

Cells were grown on plastic tissue culture flasks (75 cm², Falcon, Becton Dickinson, Franklin Lakes, NJ) in an amphibian medium supplemented with 5% fetal calf serum (Life Technologies, Paisley, United Kingdom), streptomycin (130 µg/ml) and penicillin (100 U/ml, at 28°C in 5% CO₂, atmosphere. Culture medium was changed every 2 to 3 d. Cells were split 1:10 once a week (12,13).

The experiments were performed on monolayers cultured on plastic (6-Well tissue culture plates; 35 mm or 100 mm Petri dishes; Falcon) or on rat-tail collagen-coated filter cups (0.4 µM pores, 4.7 cm², Transwell; Costar, Corning, Acton, MA). Cells cultured on plastic were seeded 1:10 and used at confluency, whereas cells cultured on filters were seeded at twice confluence density and used 10 to 15 days after seeding.

**Electrical Measurements**

The transepithelial potential difference (PD, mV) and short-circuit current (Iₑₑ, µA/cm²) were monitored on the monolayers cultured on filters. Measurements were performed in a modified Ussing chamber connected to an automatic voltage-clamp apparatus. Transepithelial electrical resistance (Ω*cm²) was calculated from the transepithelial potential difference and Iₑₑ.

**Isolation of Cellular RNA and Northern Blot Analysis**

To isolate total cellular RNA, cells were lysed in 0.5% sodium dodecyl sulfate (SDS), 100 mM NaCl, 1 mM ethylenediaminetetraacetaete (EDTA), and 20 mM Tris-HCl pH 7.5, and digested with Proteinase K (200 µg/ml; Sigma, St. Louis, MO) for 1 h at 37°C. Poly(A)+ RNA was purified by oligo(dT)-cellulose affinity chromatography. Two micrograms were electrophoresed on 0.8% denaturing glyoxal agarose gel and blotted to a Hybond-N membrane (7).

The hybridization was performed with specific PCR-generated DNA fragments obtained with the degenerated primers 5'-GGI AAT/C TGT/C TAT/C ACI TTC/T AA-3' and 5'-CGC GGA TCC CAT A/GTT T/CCT T/CCT A/GAA A/GCA-3'. The α, β, and γ subunit resulting probes were segments of 375, 381, and 369 bp, respectively. The β-actin probe is a 814-bp PvuII-HindIII fragment excised from a full-length Xenopus β-actin clone (14). The probes were radiolabeled with [α-32P]dCTP using standard procedure (Random-Primed Labeling Kit; Boehringer, Mannheim, Germany). Hybridization conditions were 16 h at 42°C in 25% formamide, 5 × SSC, 5 × Denhardt, 0.1% SDS, and 150 µg/ml of denatured salmon sperm DNA. Blots were washed at 42° to 50°C in 1 × SSC and exposed from 1 d to 1 wk at −70°C. As shown in Figure 1 (panel A), α, β, and γ transcripts were detected in A6 cells grown in filter cups with similar apparent molecular mass observed in Xenopus kidney or lung (7).

**Synthesis of α, β, and γ Subunits cRNA and Functional cRNA and Poly(A)+ RNA Expression in Xenopus Oocytes**

The α, β, and γ xENaC subunit full-length clones (in pSD5 expression vector) were transcribed into cRNA using SP6 RNA polymerase (Promega, Madison, WI).

**Figure 1.** Effect of aldosterone on α, β, and γ xENaC mRNA abundance in A6 cells grown on impermeable (plastic) or permeable (filters) support. Poly (A)+ RNA was extracted from A6 cells monolayers cultured on plastic culture dishes (lanes 1 and 2) or on filters (lanes 3 and 4), control incubated (lanes 1 and 3) or stimulated for 24 h with 300 nM aldosterone (lanes 2 and 4). Two micrograms of poly (A)+ RNA was analyzed by Northern blot with probes specific for α and β mRNA (upper panel) or β and γ mRNA (middle panel). Transcripts of 3 kb (α), 2.4 kb (β), and 3.2 kb (γ) were detected. As already shown (7), the presence of an additional β transcript was also detected. The same blot was then probed with a probe against Xenopus laevis GAP as an internal control of the amount of RNA loaded (lower panel).
For functional expression of cRNA and/or poly(A)+ RNA, 0.1 or 1 ng each cRNA subunit were injected in oocytes with or without 50 ng of poly(A)+ RNA in a total volume of 50 nl. Twenty-four hours after injection, whole-oocyte currents were measured using the two-electrode voltage clamp technique in a medium containing the following: 100 mM Na gluconate, 2 mM KCl, 1.8 mM CaCl2, 10 mM HEPES-NaOH pH 7.2, 5 mM BaCl2, and 10 mM tetraethylammonium chloride. The expressed activity was assessed by measurement of the anilamide-sensitive Na current, defined as the difference between the Na current recorded at a membrane potential of $-100 \text{ mV}$, in the absence and presence in the medium of 10 $\mu$M anilamide.

**Biosynthetic Labeling**

Cell monolayers were depleted in methionine by three 5-min washes with methionine-free and serum-free medium. For cells cultured on plastic, 200 $\mu$g of methionine-free, serum-free medium containing 1 mCi/ml $[^{35}S]$methionine (Amersham or Dupont NEN) was added on the apical surface, whereas for cells cultured on filters, the filter supporting the cells were inverted and the 200 $\mu$l were placed on the basolateral surface of the filters. Labeling was performed for 15 min at 28°C. After the incubation, cells were either washed two times with amphibian Ringer solution containing 1 mM CaCl$_2$ and then scraped in homogenization buffer, or washed once and chased for different periods in serum-free medium supplemented with 10 mM unlabeled methionine at 28°C.

**Antibodies**

Rabbit polyclonal antibodies against $\alpha$ and $\gamma$ xENaC were raised against peptides fused to the glutathione-S-transferase. The peptide consisted of amino acids 102 to 142 for $\alpha$ (region just after the first transmembrane segment) and amino acids 2 to 142 for $\gamma$ (intracytoplasmic N-terminus region). For $\beta$, we used a rabbit polyclonal antibody raised against the corresponding subunit of the rat (15). The peptide fused to the glutathione-S-transferase consisted of amino acids 559 to 636 (intracytoplasmic C-terminus region), a region sharing 47% homology with the amphibian $\gamma$ subunit.

**Immunoprecipitation**

The biosynthetically labeled cells were washed twice with Ringer plus CaCl$_2$ and scraped in 500 $\mu$l homogenization buffer (20 mM Tris-HCl, 2 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 2 mM EDTA, 6 mM mercaptoethanol and protease inhibitors). Microsomes were prepared as follows: after freezing in liquid nitrogen and thawing at 37°C, cells were sonicated, then frozen and thawed again. A 1.5-min centrifugation (20,000 × g) was performed to eliminate the nuclei and cell debris, and the supernatant was recovered and microsomes pelleted by a 50-min centrifugation at 400,000 × g. The pellet was resuspended by sonication in 400 $\mu$l homogenization buffer containing 1% Triton X-100. One last 30-min centrifugation at 20,000 × g cleared the membrane preparation, and the supernatant was recovered.

Protein recovery and $^{35}$S incorporation were determined by TCA precipitation of 5-μl aliquots of the supernatants, NaOH resuspension of the pellet and protein dosage (according to Lowry), or liquid scintillation of samples. Equal amounts of counts were submitted to immunoprecipitation with rabbit antisera.

For denaturation before incubation with antisera, SDS was added to the microsomes to a final concentration of 3.7%, and the samples were boiled for 5 min at 95°C. The preparation was preclared 20 min at 4°C with preimmune sera in 11vol of TENT 1% (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 150 mM NaCl with 1% Triton X-100), 2 mM phenylmethylsulfonyl fluoride, and 40 $\mu$l of panosorbin, and then incubated overnight at 4°C with 30 $\mu$l of the antiserum. Protein A-Sepharose was added for 1 h of incubation at room temperature, and the beads were washed four times with TENT 1% and three times with NET 2 (0.1% SDS, 2 mM EDTA, 10 mM Tris-HCl pH 7.5). The immunoprecipitated proteins were recovered in 70 $\mu$l Laemmli sample buffer.

Samples, boiled 5 min at 95°C, were loaded on 8% SDS-polyacrylamide gels. After electrophoresis, gels were treated with sodium salicylate and exposed on X-Omat S films (Eastman Kodak, Rochester, NY) at -70°C for 1 d to 3 wk.

The specificity of the three sera was tested by immunoprecipitation of the subunits expressed in pulse-labeled Xenopus oocytes, and the titer of each sera was tested by immunoprecipitation of subunits synthesized in vitro (rabbit reticulocyte lysates, Amersham) (data not shown). In A6 cells, as shown in Figure 2, the $\alpha$, $\beta$, and $\gamma$ antibodies (lane 1, upper, middle, lower panels) immunoprecipitated specifically proteins with apparent molecular weights of 75 kD, 96 kD, and 87 kD, respectively. The 94 kD for the $\gamma$ subunit immunoprecipitation (lanes 3, 4, 6, 7, 8) is non-specific and is not consistently seen (see lane 1 and 2). The corresponding preimmune sera did not immunoprecipitate any significant amount of labeled proteins (lane 2).

![Figure 2. Effect of aldosterone on $\alpha$, $\beta$, and $\gamma$ xENaC rate of protein synthesis](image-url)

Lane 5: Mr markers (67 kD and 94 kD).
Results

Effect of Aldosterone on ENaC mRNA Abundance and Rate of Protein Synthesis: Cells Grown on Plastic Culture Dishes

When poly(A)+ RNA, extracted from A6 cells grown on plastic substrate in the absence of aldosterone was injected into Xenopus laevis oocytes, channel activity was low (16) or undetectable (7). Upon aldosterone stimulation for 24 h, channel activity expressed in the Xenopus oocyte increased significantly. The data suggested that either mRNA coding for one of the three subunits is limiting and/or that aldosterone induces a regulatory protein that controls the activity of ENaC. We tested the first hypothesis by measuring ENaC mRNA abundance in these cells; as shown in Figure 1 (lane 1), α transcripts were readily detected in control cells but β and γ transcripts were undetectable. After long film exposure, a small amount of β mRNA was detected, but γ mRNA was still barely detectable. Aldosterone (24 h) (lane 2) induced a major increase in the β and the γ subunit mRNA abundance and a smaller increase in the α subunit mRNA abundance. The time-course of this effect is shown in Figure 3 and was different for each subunit, being the fastest for the γ and the slowest for the α subunit. The γ transcript was detected as early as 60 min after hormone addition, with a peak at 6 h. Significant changes in β mRNA abundance were not observed until 3 h after aldosterone addition. These data are consistent with the functional expression data reported earlier (7); however, they do not explain why Sariban-Sohraby et al. (6) did not observe an amiloride-sensitive transport in the apical membrane of these cells grown on plastic. One possible explanation is that the induction of β and γ transcripts is not accompanied by the translation of the corresponding subunit proteins in living intact cells. We have therefore measured under the same experimental conditions the rate of protein synthesis of each subunit by pulse labeling. As shown in Figure 2, the α subunit is synthesized (lane 3, upper panel) and aldosterone induced a twofold increase of its rate of synthesis after 24 h (lane 4, upper panel). No significant levels of β subunit synthesis were detected whether aldosterone was present or not (lanes 3 and 4, respectively, middle panel). Even after very long film exposures (up to 2 mo), the rate of synthesis of the β subunit remained undetectable; for the γ subunit (87-kD band), the rate of synthesis was low on cells grown on plastic and was not regulated by aldosterone (lanes 3 and 4, lower panel). On cells grown on filters, the rate of γ subunit synthesis was higher but not regulated by aldosterone (lanes 6 and 7, lower panel). By contrast, the rate of α subunit synthesis (lanes 6 and 7, upper panel) and the rate of β subunit synthesis (lanes 6 and 7, middle panel) were both upregulated by aldosterone. From these experiments, it would appear that α, β, and γ mRNA are not efficiently translated in intact living A6 cells grown on plastic and that aldosterone cannot overcome this translational block (compare A and B in Figure 3).

If transcription of ENaC β and γ subunits is the first limiting factor in the expression of sodium channel in the apical membrane of A6 cells grown on plastic in the absence of aldosterone, poly A(+) RNA extracted from nonstimulated A6 kidney cells should induce a significant channel activity in the oocyte-system, providing that adequate amounts of β and γ cRNA are coinjected to complement the poly A(+) RNA. To test this hypothesis (Figure 4), we have first expressed poly A(+) RNA from A6 cells grown on plastic in the absence or presence of
4) or stimulated for 24 h with 300 nM aldosterone (lane 2). At the end of the stimulation period, poly (A)+ RNA was extracted and 50 ng was injected in Xenopus laevis oocytes, alone (lanes 1 and 2), or complemented with β and γ cRNA (lane 4, 0.1 ng each; lane 5, 1 ng each). Lane 5: Macroscopic amiloride-sensitive sodium currents obtained with the expression of β and γ cRNA alone (1 ng each). Lane 6: Macroscopic amiloride-sensitive sodium currents obtained with the expression of α, β, and γ cRNA (1 ng each).

Figure 4. Measurements of macroscopic amiloride sensitive sodium currents expressed in Xenopus laevis oocytes injected with 50 ng of poly (A)+ RNA prepared from A6 cells cultured on plastic. A6 cell monolayers cultured on plastic were control incubated (lanes 1, 3, and 4) or stimulated for 24 h with 300 nM aldosterone (lane 2). At the end of the stimulation period, poly (A)+ RNA was extracted and 50 ng was injected in Xenopus laevis oocytes, alone (lanes 1 and 2), or complemented with β and γ cRNA (lane 4, 0.1 ng each; lane 5, 1 ng each). Lane 5: Macroscopic amiloride-sensitive sodium currents obtained with the expression of β and γ cRNA alone (1 ng each). Lane 6: Macroscopic amiloride-sensitive sodium currents obtained with the expression of α, β, and γ cRNA (1 ng each).

Effect of Aldosterone on α, β and γ xENaC mRNA Abundance and Rate of Protein Synthesis: Cells Grown on Filters, Expressing an Amiloride-Sensitive Sodium Transport

When A6 cells are grown on permeable supports, they express an amiloride-sensitive, aldosterone-induced sodium transport that can be measured by the short-circuit current method. To analyze the effect of aldosterone (300 nM) on xENaC mRNA abundance and protein synthesis, A6 cells grown on filters were serum-depleted overnight, then stimulated by increasing concentrations of aldosterone (1 nM to 1 mM) for 24 h (Figure 5). As shown previously (3,4,11,12), we observed that 300 nM of aldosterone was sufficient to elicit a near-maximal sodium transport response. This concentration was therefore selected to study the time course of aldosterone action. As shown in Figure 5E, an increase in the short-circuit current was already significant 1 h after the addition of aldosterone, reaching a maximum at 6 h (fivefold over control). Transepithelial potential difference (PD) also increased upon aldosterone stimulation (Figure 5D) but to a lesser extent (3.5-fold over control after 6 h), indicating that transepithelial resistance decreased (Figure 5F). This early change in transepithelial resistance defines the early aldosterone response, lasting 1 to 3 h (4). During this phase, it has been proposed that the hormone increases the density of open channels in the apical membrane of the epithelial cell. The simplest hypothesis for explaining the effect of aldosterone is that the hormone promotes the synthesis of new channel proteins (1). According to the well-accepted scheme of steroid action, this would imply that aldosterone induces the transcription of the channel gene subunit(s). An increase in the level of mRNA coding for the channel subunit(s) would lead to the enhanced rate of channel biosynthesis, channel assembly, and surface expression. Consistent with this idea, we found (Figure 1, lanes 3 and 4) that aldosterone led to an increase in mRNA abundance, coding for α, β, and γ subunits in cells grown on filters. Unlike the situation observed in cells grown on plastic (Figure 1, lanes 1 and 2), all three mRNA (α, β, γ) were readily detectable in the nonstimulated state. The effect of aldosterone on mRNA abundance was only significant after 24 h of stimulation (Figure 6A). Relative to the control, the effect of aldosterone was to increase three- to fourfold the abundance of each of the three messages. In contrast to the slow time course of its effect on mRNA accumulation, the effect of aldosterone on the rate of protein synthesis (Figure 6B) had a much faster time course. It was very large for the α subunit (12-fold increase over control), significantly lower for the β subunit (fourfold over control), and there was no significant change in the rate of synthesis for the γ subunit, despite a significant increase in its mRNA abundance. The time course of induction of mRNA is shown in Figure 6A; there was no significant increase in mRNA accumulation, the effect of aldosterone on the rate of protein synthesis (Figure 6B) had a much faster time course. It was very large for the α subunit (12-fold increase over control), significantly lower for the β subunit (fourfold over control), and there was no significant change in the rate of synthesis for the γ subunit, despite a significant increase in its mRNA abundance. The time course of induction of mRNA is shown in Figure 6A; there was no significant increase in mRNA abundance during the early phase of aldosterone action. By contrast (Figure 6B), the rate of protein synthesis was rapidly and differentially regulated by aldosterone. As early as 1 h after hormone addition, the rate of synthesis of the α subunit was significantly higher than that of the controls and of the β and γ subunits. This differential effect was maintained at 3 h and 6 h after hormone addition. The effect on the rate of synthesis of the β subunit became significant only after 6 h of hormone treatment, whereas the effect of aldosterone on the rate of synthesis of the γ subunit never reached the level of significance.
Figure 5. Dose-response analysis and time course of the effects of aldosterone on sodium transport in A6 cells cultured on filters. A6 cell monolayers cultured on filters were stimulated for 24 h with increasing doses of aldosterone and the effects on transepithelial potential differences (PD) (Panel A), short-circuit current ($I_{sc}$) (Panel B), and transepithelial resistance ($R$) (Panel C) were monitored. The values are expressed as the percentage of the maximal response obtained. A6 cell monolayers cultured on filters were stimulated with 300 nM aldosterone and the effects on transepithelial potential differences (PD) (Panel D), short-circuit current ($I_{sc}$) (Panel E), and transepithelial resistance ($R$) (Panel F) were monitored over 24 h. The values are expressed as fractional changes normalized to the time 0.

Turnover of the Newly Synthesized Pool of $\alpha$, $\beta$, and $\gamma$ ENaC

The increase in the rate of synthesis of $\alpha$ and $\beta$ ENaC subunit could lead to an increase in the number of conducting channels in the apical membrane, provided that the rate of degradation of each subunit is not increased by aldosterone. To rule out this possibility, we examined the turnover rate of ENaC subunits in A6 cells grown on filters. A6 cells were first labeled with $[^{35}S]$ methionine for 15 min, then chased with unlabeled methionine for various periods of time, up to 6 h. The subunit turnover was quantified by densitometric scanning, and the rate constant for degradation was measured in the presence or absence of aldosterone. The calculated half-lives were short, less than 1 h for the $\alpha$ subunit and close to 1 h for the $\beta$ and $\gamma$ subunit (mean values in minutes in Table 1). None of the subunits’ turnover rates were affected by aldosterone treatment. No change was observed when the cells were preincubated overnight with aldosterone (300 nM) or when they were pulsed and then chased in the absence or presence of the hormone. In conclusion, the turnover rate of each subunit is rapid (less than 1 h) and not significantly affected by aldosterone treatment.

Discussion

Dual Effect of Aldosterone

Amiloride-sensitive, electrogenic sodium reabsorption is mediated by two transport proteins involving the apical influx of sodium into the cell via ENaC and the basolateral extrusion of sodium by Na,K-ATPase. As recently reviewed by Verrey and Beron (18), the action of aldosterone on ENaC and Na,K-ATPase is twofold:

(1) to rapidly increase the activity of the two transporters.
At the end of the stimulation period, poly (A)+ RNA cultured on filters. A6 cell monolayers cultured on filters were control abundance were assessed by densitometric scanning of the gels. and 2 i.g were analyzed by Northern blot with probes specific for α, β, and γ subunit and for -actin. mRNA abundance of control incubated cells was arbitrarily defined as 1. (A) At the end of the stimulation period, cells were incubated or stimulated 1, 3, 6, or 24 h with 300 nM aldosterone. (B) At the end of the stimulation period, cells were immunoprecipitated from same number of counts incorporated in the cell lysates and resolved by SDS-PAGE. The fractional changes in the number of channel proteins in the membrane can increase as a result of the synthesis and/or insertion of new channels (1). There are three possible basic mechanisms: first, the number of channel proteins in the membrane can increase as a result of the synthesis and/or insertion of new channels into membranes; second, channels already in the membrane in an inactive form could be activated by a covalent modification of the channel protein; and third, the channel that spontaneously fluctuates between open and closed states could remain open for a greater fraction of their time. These three basic mechanisms are not mutually exclusive. Our data provide the

```
Figure 6. Time-course of the effect of aldosterone on α, β, and γ ENaC mRNA abundance and rate of protein synthesis in A6 cells cultured on filters. A6 cell monolayers cultured on filters were control incubated or stimulated 1, 3, 6, or 24 h with 300 nM aldosterone. (A) At the end of the stimulation period, poly (A)+ RNA was extracted and 2 μg were analyzed by Northern blot with probes specific for α, β, or γ subunit and for -actin. The fractional changes in mRNA abundance were assessed by densitometric scanning of the gels. Values for mRNA abundance were normalized by those obtained for -actin. mRNA abundance of control incubated cells was arbitrarily defined as 1. (B) At the end of the stimulation period, cells were 15-min pulse-labeled with 35S methionine. α, β, and γ subunits were immunoprecipitated from same number of counts incorporated in the cell lysates and resolved by SDS-PAGE. The fractional changes in the rate of subunit synthesis were assessed by densitometric scanning of the gels and are expressed with values obtained for control incubated cells arbitrarily defined as 1.
```

This effect takes place during the early phase of aldosterone action after a latency of < 1 h.

(2) to increase the number of the transporters after a latency of > 3 h.

The dual effect of aldosterone appears to be mediated by transcriptional mechanisms because the early and late phases of aldosterone action are both fully inhibited by actinomycin D. This view is well-supported in the A6 kidney cell model for the aldosterone-dependent regulation of Na,K-ATPase. In this model, the early activation of Na,K-ATPase is well-documented (13,19), as well as the late accumulation of the enzyme preceded by a transcriptional activation of Na,K-ATPase genes (11,14).

In the study presented here, we show that aldosterone induces a late accumulation of α, β, and γ ENaC mRNA. Because we have not yet measured the rate of transcription of these genes, we do not know whether the increased abundance of mRNA is mediated by a direct transcriptional effect and/or by an effect on mRNA turnover. The effects of aldosterone on ENaC mRNA abundance in a cultured kidney cell line described in this study are similar to those observed in rabbit CCD cells for γ ENaC (20) and somewhat larger than those reported in rat kidney (21). In rat inner medullary collecting ducts, either glucocorticoid hormone or mineralocorticoid hormone increased the amount of α-rENaC subunit mRNA but had no effect on the mRNA level of the β-rENaC or γ-rENaC subunits (22).

Interestingly, the effect of aldosterone on the rate of protein synthesis was not directly proportional to the effect on mRNA abundance. The discrepancy between the two effects was particularly significant for the α and the γ subunit. We have no explanation for these observations. They suggest, however, that aldosterone, besides its classical transcriptional effect, may control gene expression by different mechanisms (see below).

It is interesting to note that aldosterone has a much smaller effect on the rate of synthesis of the β and no effect on the rate of synthesis of the γ subunit. We currently have no explanation for this differential effect of aldosterone on the rate of subunit synthesis. It is important to note that it appears that the rate of synthesis of the α subunit is much larger than the β or γ subunit, both in the control and stimulated states. These differences may reflect the stoichiometry of ENaC, which, at present, has not been elucidated. We recently proposed that the heteromultimeric protein is composed of more than three subunits (23); our data are compatible with the stoichiometry in which there are more α subunits than β and/or γ subunits in the channel complex.

**Effect of Aldosterone on the Synthesis of the α Subunit of ENaC: Implication for the Early Sodium Transport Response**

Apical sodium permeability increases in many aldosterone-responsive epithelia, and there is an excellent correlation between the presence of amiloride-sensitive sodium channels and the ability of aldosterone to enhance transport through those channels (1). There are three possible basic mechanisms: first, the number of channel proteins in the membrane can increase as a result of the synthesis and/or insertion of new channels into membranes; second, channels already in the membrane in an inactive form could be activated by a covalent modification of the channel protein; and third, the channel that spontaneously fluctuates between open and closed states could remain open for a greater fraction of their time. These three basic mechanisms are not mutually exclusive. Our data provide the
first evidence that aldosterone already increases the rate of synthesis of the α subunit of ENaC 60 min after addition, just at the time where sodium transport begins to rise (Figure 7), raising the possibility that de novo synthesis of channel may mediate in part the early increase in sodium transport. In the oocyte expression system, we have observed that the α subunit is the limiting factor in the assembly and/or export of newly synthesized channels to the plasma membrane (10). If this is also true in A6 kidney cells, the effect of aldosterone on the synthesis of α subunits could indeed be also limiting in the action of aldosterone on transepithelial electrogenic sodium transport during the early phase of aldosterone action. The rapid turnover of the α subunit (less than 50 min) is compatible with a possible rapid insertion of newly synthesized ENaC molecules in the apical membrane of A6 cells, mediating part or all of the early sodium transport response. Because aldosterone does not change the turnover of ENaC subunits, one could predict a direct and proportional increase in ENaC molecules during this phase, assuming that the rate of protein synthesis is paralleled by a similar rate of assembly, export, and insertion into the apical membrane. This implies that the density of ENaC molecules in the apical membrane would increase proportionally to the rate of protein synthesis. Unfortunately, using a variety of surface-labeling protocols, we have not been able to detect ENaC molecules directly in the apical membrane of these cells (data not shown). This is probably because the present methodology does not allow the detection of such low copy number protein, which is estimated to be a few hundred molecules in the apical membrane of one cell (24). In the study presented here, we have not addressed the question of whether aldosterone could regulate the assembly, the oligomerization of the protein in the endoplasmic reticulum, and its transport to the plasma membrane; each step could be a site of hormonal regulation. Unfortunately, our antibodies do not allow us to address these questions because we are not able to immunoprecipitate the assembled complex in a native configuration (non-denaturing).

In the toad bladder, the apical sodium permeability increases during both the early and the late responses to aldosterone. It was postulated that the mechanisms through which aldosterone is exerting its effect are different in the two phases; in the early phase, the enhanced permeability is caused by activation of channels already present in the apical membrane and accessible to luminal modifiers (25–27). During the late phase, the increased permeability is sensitive to thyroid hormone and sodium butyrate (27) and may be caused by de novo synthesis of channels (28).

In rat cortical collecting ducts, using physiological manipulations allowing plasma aldosterone to vary within large margins (animals from a normal diet to a low-salt diet), channel activity could be induced in animals fed a low-salt diet (high plasma aldosterone) for at least 48 h. By contrast, animals on a normal diet (low plasma aldosterone) did not show any significant channel activity (29). The effect was interpreted as

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Pulse-Chase</th>
<th>Pulse-Chase</th>
<th>Pulse-Chase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–/– Aldo</td>
<td>+/- Aldo</td>
<td>+/- Aldo</td>
</tr>
<tr>
<td>α</td>
<td>0.032</td>
<td>0.022</td>
<td>0.027</td>
</tr>
<tr>
<td>β</td>
<td>0.016</td>
<td>0.016</td>
<td>0.015</td>
</tr>
<tr>
<td>γ</td>
<td>ND</td>
<td>0.018</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Table 1. Rate constants of degradation and half-lives of α, β, and γ xENaC determined by pulse-chase experiments

A6 cells cultured on filters were 15-min pulse-labeled with 35S methionine and then chased in the presence of excess cold methionine for 0, 15, 45, 120, or 180 min, or 24 h. The experiments were performed in the absence of aldosterone (–/–) or in the presence of the hormone (300 nM), 24 h before and during the pulse (+/–) or during the whole experiment (+/+). α, β, and γ subunits were immunoprecipitated from same amount of counts incorporated in the cell lysates and resolved by SDS-PAGE. The percentage of incorporated label remaining, determined by densitometric scanning of the gels, was reported on a semilogarithmic scale. As a starting point, we used the incorporated label at 15 min chase. The slope of the linear regression (k) (min⁻¹) was calculated, as well as the corresponding half-lives (t½) (minute). The values are the means of three distinct experiments.

![Figure 7](image-url) Figure 7. Effects of aldosterone (300 nM) on A6 cells grown on filters: summary of the results obtained at the levels of short-circuit current (Isc), α mRNA abundance, and a rate of α subunit protein synthesis.
mainly a result of an increase in a number of channels per patch. This approach, however, does not allow us to distinguish among the three basic mechanisms discussed above. These data apparently contrast with those described by Kemendy et al. (8), in whose study it was shown that aldosterone increased the open probability (Po) of sodium channel in the amphibian A6 kidney cell line. As discussed by Pacha et al., the difference could be quantitative rather than qualitative (29). In favor of the concept of activation, preformed apical channels in A6 cells come from studies in which aldosterone did not increase the number of membrane amiloride binding sites (30) or in the level of amiloride-sensitive channel assessed by idiotypic antibody (31). Unfortunately, in the latter studies, it is not possible to rule out that amiloride binding protein rather than ENaC molecules were measured by these assays.

There is no easy explanation to reconcile these apparently contradictory studies until a more sensitive method to quantify precisely the number of channel molecules present in the apical membrane is developed to approach this important issue directly. Without such a method, it will remain difficult to reconcile the conclusions of studies performed in various experimental systems that support the concept of either activation of preformed silent channels in the apical membrane or a switch from a low Po to a high Po gating mode with the present data, which support an increase in the number of channel molecules in the apical membrane.

**Effect of Aldosterone on ENaC Gene Expression:**

**Transcriptional versus Translational Mechanisms and State of Epithelial Differentiation**

An important factor was, however, never considered in previous studies: the extremely rapid turnover of ENaC protein. The turnover of transport protein is usually slow (for Na,K-ATPase in A6 cells, typically around 18 h [12]). A turnover of less than 60 min as shown here for the amphibian ENaC would allow quite a rapid modulation of the expression at the cell surface. Such a regulatory system may be ATP-consuming, but ENaC is a low copy number protein (at least 1000-fold less abundant than Na,K-ATPase), thereby minimizing energy cost. The experiments presented here indicate that the mechanisms involved may be different than previously thought: we would like to propose that a major translational effect of aldosterone plays a distinct role during the early phase of hormonal action. Interestingly, this effect is only observed in cells that are fully differentiated and express an aldosterone-dependent electrogenic sodium transport. In a less differentiated state, the abundance of mRNA coding for the β and γ subunits is under strong aldosterone control, but this transcriptional or posttranscriptional effect on mRNA abundance is not sufficient to induce channel activity at the cell surface, apparently because these RNA are not translated into their protein in the intact cell. It is worthwhile to note that in tissues in which cell turnover is high, such as rat colon (32) or avian intestine (33), a major effect of aldosterone on β and γ ENaC mRNA abundance has been observed. In these experiments, the rate of protein synthesis was, however, not measured and it is not precisely known when and where during colonic crypt cell terminal differentiation the sodium channel protein is synthesized, assembled, and exported to the apical membrane. By immunocytochemistry, the α ENaC was clearly localized exclusively in the apical membrane of the surface epithelial cells, whereas by in situ mRNA, molecules were detected in cells in the middle of the crypt (15,32). It could very well be that in kidney cells in culture, with a relatively high rate of replication (A6 cells on plastic) as in rat colon cells, aldosterone has a major transcriptional effect on β and γ subunits. In terminally differentiated kidney cells, where the rate of replication is small, α, β, and γ mRNA would be constitutively expressed and minimally controlled by aldosterone. The major effect of aldosterone in these cells would be at the translational and/or posttranslational levels.

Translational regulation is a well-established mechanism in prokaryotic and eukaryotic cells. Translational regulation by steroid hormones has also been proposed recently, and a steroid modulatory element in the 5' untranslated region of the myelin basic protein mRNA has been identified (34). We do not know whether the observed effect on the rate of synthesis of α ENaC is due to a direct interaction of the aldosterone-steroid receptor complex with the translational machinery and/or whether it is mediated by regulatory proteins that are transcriptionally induced during the latent period by aldosterone.

**Acknowledgments**

We thank Nicole Skarda-Coderey for her secretarial work and François Verrey for reading and improving this manuscript. This article was funded by the Swiss National Fund for Scientific Research, Grant 31-43384.95.

**References**

9. Palmer LG, Frindt G: Amiloride-sensitive Na channels from the

---

Effect of Aldosterone on ENaC Protein Synthesis
apical membrane of the rat cortical collecting tubule. *Proc Natl Acad Sci USA* 83: 2767–2770, 1986