Aldosterone Modulates Sodium Kinetics of Na,K-ATPase Containing an $\alpha 1$ Subunit in A6 Kidney Cell Epithelia

Jörg Beron, Luca Mastroberardino, Adrian Spillmann, and François Verrey*

Institute of Physiology, University of Zürich, CH-8057 Zürich, Switzerland

Submitted December 27, 1994; Accepted February 7, 1995

Monitoring Editor: Guido Guidotti

Short-term aldosterone (10^{-6} M, 2.5 h) induces in A6-C1 cell epithelia an increase in Na transport, which is due to the in situ activation of the apical Na channel and, presumably, the basolateral Na pump (Na,K-ATPase). We have now directly measured the effect of aldosterone on the transport activity of endogenous Na pumps and hybrid Na pumps containing an exogenous $\alpha 1$ subunit by measuring the pump current (I_p) across epithelia apically permeabilized with amphotericin B. Aldosterone (2.5 h) had no significant early effect on the maximal I_p , nor on the Na concentration required for half-maximal activation. In contrast, it increased the I_p at physiological intracellular Na concentrations (1.7-fold at 5 mM Na). This effect was blocked by the protein synthesis inhibitor cycloheximide. Hybrid pumps containing the transfected cardiotonic steroid-resistant $\alpha 1$ subunit of *Bufo marinus* were also stimulated by aldosterone (2.5 h). A long aldosterone treatment (4 days) increased the maximal I_p produced by the endogenous pumps 1.5 to 2.1-fold. In conclusion, aldosterone acts on Na pumps containing an $\alpha 1$ subunit in two ways. During its early phase of action it stimulates their transport activity by increasing their apparent Na affinity at physiological intracellular Na concentrations. In the long term it produces an increase in the maximal transport capacity, which corresponds to the known increase in the number of Na pumps.

INTRODUCTION

Mineralocorticoid hormones control the final adjustment of urinary Na concentration by regulating the Na reabsorption across distal nephron epithelia via transcriptionally mediated mechanisms. A major site of this regulated transport is the mammalian cortical collecting duct (principal cells), and the *Xenopus laevis* A6 cell line, which forms tight epithelia on filter supports, is a widely used model for its study. Sodium is reabsorbed across these cells by a two-step mechanism. First, Na enters the cells from the mucosal side through the highly selective, epithelial Na channel, a step which is generally considered as rate limiting. Second, Na ions are extruded from the cells across the basolateral membrane by the Na pump (Na,K-ATPase), which also provides the elec-

trochemical driving force for the apical Na influx (Rossier and Palmer, 1992; Verrey, 1995; and refs. therein).

Short-term aldosterone (≤ 3 h) increases the activity of preexisting apical Na channels, via as yet unidentified transcriptionally regulated mediator(s) (Garty, 1994; Verrey, 1995). In A6 cells, this effect has been shown to correspond to an increase in channel open probability (Kemendy *et al.*, 1992). In contrast, at later stages of hormone action (≥ 3 h), it appears that the total number of active Na channels is increased (Pácha *et al.*, 1993).

The kinetic properties of the Na,K-ATPase, which at physiological intracellular Na concentrations functions below its half-maximal activity and shows a steep Na activation curve (positive cooperativity), could accommodate large changes in Na transport rate. However, several reports have supported the hypothesis that aldosterone exerts, besides its apical effect, a coordinated stimulatory effect at the level of

* Corresponding author: Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.

the Na,K-ATPase (Rossier and Palmer, 1992; Verrey, 1995; and refs. therein). Such an effect would maintain the driving force for apical Na entry and keep the intracellular Na concentration relatively constant despite large changes in Na transport rate (Schultz, 1981; Palmer and Speez, 1986; Bastl and Hayslett, 1992; and refs. therein). This could be of particular importance at very low luminal Na concentrations and also in the case of high transport rates because in the latter case, besides a decrease in driving force, an increase in intracellular Na would produce a feedback inhibition at the level of the apical Na channels (Frindt *et al.*, 1993; Silver *et al.*, 1993).

It has been previously shown by measuring ouabain binding rates in the absence of extracellular K that Na pumps are activated by a short-term aldosterone treatment in A6 cells (Pellanda *et al.*, 1992; Beron and Verrey, 1994a). By immunoprecipitating cell-surface-labeled Na,K-ATPase, we have also shown that this effect is not mediated by the translocation of a large number of pumps to the cell surface (Beron and Verrey, 1994a). Because no kinetic parameters of the Na pumps involved in the early response were determined, these approaches did not allow us to discriminate between several possible mechanisms. For example, the question of whether the effect was due to the appearance of a (small) subpopulation of pumps with different kinetics, eventually containing another α subunit isoform, remained open.

To directly measure the impact of aldosterone treatment on the Na pump transport activity at defined intracellular Na concentrations, we have now measured the current generated by the basolateral pumps (I_p)¹ in epithelia apically permeabilized to monovalent ions with amphotericin B (Kirk *et al.*, 1980; Lewis and Wills, 1983). To test whether the observed regulation could be due to an effect on pumps containing the $\alpha 1$ subunit we generated A6-C1 cell lines stably expressing an exogenous $\alpha 1$ subunit that confers an intermediate level of cardiotoxic steroid resistance to hybrid pumps (containing an endogenous β subunit). This system allowed us to specifically test the regulation of the function of pumps containing the exogenous $\alpha 1$ subunit.

MATERIALS AND METHODS

Cell Culture

A6 cells from the A6-C1 subclone (passages 109 to 122) were cultured on polycarbonate filters (Transwell, 0.4- μ m pore size, 4.7 cm², Costar, Cambridge, MA) coated with a thin collagen layer, as previously described (Beron and Verrey, 1994a). Experiments were performed on epithelia cultured for a total of 15–22 days. For the first 10 to 17 days the epithelia were cultured in medium containing bicarbonate and 10% fetal bovine serum and were transferred to

serum- and bicarbonate-free medium at least 4 days before the experiment. Aldosterone, if required, was added to a final concentration of 10^{-6} M to both compartments. We routinely use this concentration of aldosterone, at which both high and low affinity adrenal steroid receptors are occupied, because it produces a maximal response at the level of Na transport and at that of Na,K-ATPase mRNA accumulation (Verrey, 1990). Vehicle (ethanol 0.01%) was given to control epithelia.

The cell line TBM-54, which is derived from the urinary bladder of the toad *Bufo marinus*, and amphibian medium supplemented with 10 mg/l insulin, 1 nM dexamethasone, and 5% fetal bovine serum were gifts from B.C. Rossier (Institute of Pharmacology and Toxicology, University of Lausanne, Switzerland) (Handler *et al.*, 1979). Filter cultures were made as for A6-C1 cells and cultured for 3 wk in amphibian medium before changing, 1 day before the experiment, to the serum- and bicarbonate-free medium (the same as for A6-C1 epithelia).

A6 Cell Lines Expressing the $\alpha 1$ Subunit of the *Bufo marinus* Na,K-ATPase ($\alpha 1$ TBM)

The $\alpha 1$ TBM cDNA (Jaisser *et al.*, 1992) was transferred to the eukaryotic expression vector pcDNAI (Invitrogen, San Diego, CA). The day before transfection, nonconfluent A6-C1 cells were passaged at a density of 2×10^5 cells per 9.6 cm² dish. Plasmid DNA (2 μ g) mixed with 6 μ l Lipofectamine (Life Technologies, Gaithersburg, MD) in serum-free medium was given to prewashed cells in a final vol of 1 ml. After an overnight incubation, the medium was removed and replaced by 2 ml of medium containing 10% fetal bovine serum. The next day, the cells were passaged and seeded on 78-cm² culture dishes. Selection for cardiotoxic steroid-resistant cells was started 1 day later by the addition of ouabain at a final concentration of 20 μ M. The ouabain-supplemented culture medium was changed every 3 days and individual colonies were isolated after 2–3 wk by “ring cloning”. Culture conditions of the cardiotoxic steroid-resistant cell lines were as for A6-C1 cells and ouabain (20 μ M) was only given to cells cultured on dishes. Experiments were performed between passages 5 and 20 after ring cloning. To enhance the expression level of the $\alpha 1$ TBM subunit, sodium butyrate was given over a period of 4 days (2 or 5 mM). This treatment did not significantly interfere with the early and late effects of aldosterone on short-circuit current, in contrast to the situation in the toad bladder in which butyrate specifically inhibits the late response (Truscello *et al.*, 1983).

Electrical Measurements

Transepithelial electrical measurements were performed as previously described on monolayers cultured on Transwell filtercups in a modified Ussing chamber using an automatic voltage-clamp device (Paccolat *et al.*, 1987; Verrey, 1994). Measurements of the transepithelial potential difference and short-circuit current were routinely performed in serum-free *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered medium, before apical permeabilization.

For the measurement of the I_p , epithelia were permeabilized apically to monovalent ions using amphotericin B. They were kept throughout the experiment in symmetrical buffers containing 5 mM Ba and in short-circuit configuration (De Kruijff *et al.*, 1974; Kirk *et al.*, 1980; Lewis and Wills, 1983; Palmer and Speez, 1986; Broillet and Horisberger, 1991; Welling *et al.*, 1993). Briefly, after a pretreatment of 2.5 h with aldosterone (10^{-6} M) or diluent, monolayers were washed twice on both sides with 2 ml Na-free buffer (buffer A contained in mM: 5 Ba(OH)₂, 25 HEPES, 98 potassium gluconate, 1 K₂HPO₄, 1 calcium gluconate, 1 MgCl₂, 10 glucose, pH 7.35). This step and the following ones were made in the absence of hormone. Filter cultures were then incubated for 30 min at 28°C with 2 ml buffer A supplemented with 20 or 40 μ g/ml amphotericin B (Fungizone, Life Technologies) in the apical compartment and 2.5 ml of

¹ Abbreviations used: I_p , Na pump current; $\alpha 1$ TBM, Na,K-ATPase $\alpha 1$ subunit of the toad *Bufo marinus*.

buffer A in the basolateral compartment. Thereafter, rings were transferred to the modified Ussing chamber with fresh, identical buffers and maintained at 26°C. Epithelia were kept voltage clamped at 0 mV and the injected current (corresponding to the short-circuit current) was monitored continuously. No cardiotoxic steroid-sensitive current could be measured in the absence of Na and the transepithelial resistance was 2000–5000 $\Omega \times \text{cm}^2$. Activation of the Na pump by defined intracellular Na concentrations and in the presence of symmetrical media was performed by sequentially replacing, in the basolateral and in the apical compartments, precise volumes of buffer A with the Na-containing, K-free buffer B, without interrupting the voltage clamp (buffer B was identical to buffer A but contained sodium gluconate and Na_2HPO_4 instead of potassium gluconate and K_2HPO_4). Most of the current induced by the medium change was inhibitable by cardiotoxic steroids and could be considered as I_p because the experiments were performed on short-circuited epithelia maintained in symmetrical buffers (absence of electrochemical driving forces) and in the presence of barium (inhibition of basolateral K recirculation) (see recordings in Figures 2 and 5). Amiloride (10 μM), which inhibited approximately 90% of the short-circuit current in intact epithelia (Verrey, 1994), did not modify the Na-activated current across epithelia treated apically with amphotericin B, confirming that the majority of the cells were apically permeabilized.

To measure the activation of the Na pumps by various Na concentrations in the same epithelium, stepwise increases of the Na concentration (see above) were performed. When long experiments with numerous steps were performed (see Figure 1A) the current became unstable after approximately 30 min and a “pump activity run down” with an absence of further increase in I_p above 50 mM Na was observed. Therefore, measurements were repeated with only six different Na concentrations (in mM: 0, 2.5, 10, 20, 40, 60). Sigmoidal curves were fitted to the experimental points and values of the maximal I_p ($I_{p(\text{max})}$), the Na concentration required for half-maximal activation ($K_{0.5}$), and the Hill coefficient (n) were derived using a nonlinear regression analysis routine (InPlot v.4, Graph-PAD, San Diego, CA). The values of $I_{p(\text{max})}$ obtained by measuring the cardiotoxic steroid-inhibitable current after direct activation of the pumps with 90 mM Na (see below) were not significantly different from those obtained by this curve fitting (compare second paragraph of RESULTS with Table 2). According to the parameters derived by curve fitting, 97% of the $I_{p(\text{max})}$ should be reached at 90 mM Na.

For the precise measurement of the (cardiotoxic steroid-inhibitable) I_p induced by a single Na concentration, such as for the determination of the aldosterone effect on the pump function, the I_p was blocked with ouabain. In this case, Na-containing medium (to a final Na concentration of 2.5, 5, 10, or 20 mM) was given as described above after a 10-min equilibration period in short-circuit configuration. For 90 mM Na, a complete buffer replacement was performed. The I_p was inhibited 4 min after the addition of Na (for Na concentrations ≤ 10 mM) by the addition of 1 mM ouabain (from a 10 mM stock made in buffer with the corresponding Na concentration) to the basolateral compartment. The difference in current before and 1 min after addition of ouabain was taken as cardiotoxic steroid-sensitive I_p .

The effect of aldosterone at the level of the hybrid pumps containing $\alpha 1\text{TBM}$ (which confers to hybrid pumps an intermediate level of cardiotoxic steroid resistance) was measured as described above for the endogenous pumps but in the presence of 50 μM strophanthidin in the basolateral chamber. This concentration was required to block the endogenous pumps activated by a 5 mM Na step of 4 min.

To determine the K_i of strophanthidin in epithelia of A6-C1 cells, in A6-C1 cell lines expressing the $\alpha 1\text{TBM}$ subunit, and in *Bufo marinus* TBM-54 cells, apical permeabilization was performed in the Ussing chamber with 40 $\mu\text{g}/\text{ml}$ amphotericin B, in Na-containing buffer C and in open-circuit configuration for 10 min (buffer C contained in mM: 5 BaCl_2 , 10 HEPES, 90 sodium gluconate, 1

K_2HPO_4 , 1 calcium gluconate, 1 MgCl_2 , 12.5 glucose, 4 KOH, 4 potassium gluconate, pH 7.4). After switching to the short-circuit configuration and equilibration of the I_p , increasing concentrations of cardiotoxic steroids were added to the basolateral compartment. Equations describing hyperbolic models for one or two independent binding sites were used to fit curves to the data (InPlot v.4, Graph-PAD). The K_i and the amount of current carried by each of the two Na pump populations were derived from the fits. A limiting factor was the time required for reaching an equilibrium at low cardiotoxic steroid concentrations. Therefore, the aglycone strophanthidin, which has a faster on and off rate than the cardioglycoside ouabain, and also a higher dissociation constant, was preferred (Forbush, 1983). However, the lowest strophanthidin concentration used (0.4 μM) still inhibited approximately two-thirds of the I_p of the A6-C1 Na,K-ATPase. Another limitation was the solubility of strophanthidin (given from a 10 mM stock solution made in ethanol) such that ouabain (2 mM) was taken for maximal inhibition.

Western Blot

A polyclonal antibody to a peptide (SEHGKKKGKKGKDRDME) that corresponded to the amino acid residues 16 to 33 of $\alpha 1\text{TBM}$ (Jaisser *et al.*, 1992) and that was synthesized on a MAP4 branching lysine core (Posnett and Tam, 1989) was raised in rabbits. To prevent the cross-reaction of this antibody with the A6 (*Xenopus laevis*) $\alpha 1$ subunit, it was preabsorbed with soluble and immobilized fusion protein containing the *X. laevis* $\alpha 1$ subunit N-terminal region (Ackerman and Geering, 1990). Microsomal proteins were prepared from the different cell lines and equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Kleyman *et al.*, 1989). Western blotting and signal detection using a chemiluminescence system were as described previously (Beron and Verrey, 1994a).

RESULTS

I_p : A Measure of the Na,K-ATPase Transport Function at Defined Intracellular Na Concentrations

To directly measure the transport activity of the basolateral Na pumps in A6 epithelia at defined intracellular Na concentrations, the apical membrane was permeabilized to monovalent ions with amphotericin B (De Kruijff *et al.*, 1974; Lewis and Wills, 1983; Palmer and Speez, 1986). Symmetrical buffers were used and the epithelia were maintained voltage clamped at 0 mV to minimize electrochemical forces that could drive currents through basolateral conductances, exchangers, and cotransporters. Low Cl concentrations were chosen to avoid cell swelling and the consecutive opening of basolateral conductances (Broillet and Horisberger, 1991; Welling *et al.*, 1993). Furthermore, Ba was added to block K channels and thereby avoid K recirculation across the basolateral membrane (Broillet and Horisberger, 1991; Granitzer *et al.*, 1991). In these conditions nearly all of the current activated by Na was inhibitable by the cardiotoxic steroid ouabain and is expected to correspond to the direct electrogenic action of the Na pump, namely to the transfer of one unit charge per cycle (three Na are exchanged against two K) (De Weer, 1992; and refs. therein). Figure 1, which shows an original recording (A) and the corresponding Na activation curve (B) for a stepwise, symmetrical increase in Na concentration,

illustrates the effect of the Na concentration on the current.

Aldosterone Increases the I_p Activated by Low Na Concentrations

To compare control and aldosterone-treated (2.5 h) epithelia, a series of experiments with only five Na concentration steps (2.5, 10, 20, 40, 60 mM) was performed because in long experiments with more Na concentration steps a "run down" of the response to high Na concentrations was observed (see MATERIALS AND METHODS). $I_{p(max)}$, Na concentrations required for half-maximal activation ($K_{0.5}$), and Hill coefficients (n) were derived from the experimental points by curve fitting. These parameters, which in this case are essentially representative of the pump function at medium and high Na concentrations (see DISCUSSION), were not significantly different in the two conditions: $I_{p(max)} = 5.7 \pm 0.7$ for control versus $5.7 \pm 0.9 \mu A/cm^2$ for aldosterone-treated epithelia; $K_{0.5} = 21.2 \pm 1.2$ versus 19.4 ± 1.8 mM; $n = 2.4 \pm 0.2$ versus 2.4 ± 0.1 (mean $\pm$ SE, $n = 6$). The values for the $K_{0.5}$ and n are well within the variability of data obtained from different systems and similar to those found by Lewis and Wills (Lewis and Wills, 1983) using a similar approach on rabbit urinary bladder ($K_{0.5} = 14$ mM, $n = 2.8$). Although the overall shape of the Na activation curve was similar in control and aldosterone (2.5 h)-treated epithelia, we observed at low Na concentrations (≤ 10 mM) a clear tendency toward higher I_p in the latter ones.

To confirm and quantify this putative effect of aldosterone (2.5 h, 10^{-6} M) on the I_p activated by low Na concentrations, we directly measured the cardiotonic steroid-inhibitable I_p at the Na concentrations of interest. When the I_p activated by the single Na concentra-

Table 1. Short-term aldosterone (2.5 h; 10^{-6} M) increases I_p at low Na concentrations in epithelia of A6-C1 cells apically permeabilized with amphotericin B

Na (mM)	Mean $I_p \pm$ SE ($\mu A/cm^2$)		n	% increase
	control	aldosterone		
2.5	0.045 ± 0.006	0.072 ± 0.009	15	$70 \pm 12^{**}$
5	0.17 ± 0.02	0.27 ± 0.02	14	$66 \pm 9^{**}$
10	0.70 ± 0.09	0.89 ± 0.09	4	$29 \pm 4^{**}$
20	2.20 ± 0.10	2.28 ± 0.02	5	5 ± 4
90	6.61 ± 0.43	6.91 ± 0.43	4	5 ± 3
2.5(+cyclo)	0.024 ± 0.004	0.027 ± 0.006	4	12 ± 10

I_p were activated with the indicated concentrations of Na. Cycloheximide (0.5 $\mu g/ml$) (+ cyclo) was given simultaneously with aldosterone. The I_p values represent means ($\pm$ SE from n paired experiments) of the current, inhibited within 1 min by 1 mM ouabain. Paired two-tailed t -test of test vs. control: $^{**}P < 0.01$.

Figure 1. Sodium-activation of I_p across A6-C1 epithelia apically permeabilized with amphotericin B. (A) Original recording of the I_p activated by steps of increasing Na concentrations in an A6-C1 control monolayer (see MATERIALS AND METHODS). Numbers indicate the Na concentration in mM. (B) Concentration-response relation: a sigmoidal curve was fitted to the data of panel A. The mean $I_{p(max)}$, $K_{0.5}$, and Hill coefficient derived from six independent experiments were $5.7 \mu A/cm^2$, 21.2 mM Na, and 2.4, respectively, for control epithelia. The results were not significantly different for short-term aldosterone-treated (10^{-6} M, 2.5 h) epithelia (see RESULTS).

tion step reached a maximum (after 4 min for the low Na concentrations), 1 mM ouabain was added basolaterally to block the Na,K-ATPase. The current inhibited within 1 min after the addition of the inhibitor was taken as I_p . Original recordings of experiments with 2.5, 5, 10, and 20 mM Na are shown in Figure 2 and the results are summarized in Table 1. Significant differences in the I_p between control and short-term aldosterone-treated monolayers were observed at Na concentrations ≤ 10 mM (70% increase at 2.5 mM Na),

Figure 2. Short-term aldosterone (10^{-6} M, 2.5 h) increases the I_p at low intracellular Na concentrations. Original recordings of I_p activated by the indicated Na concentrations (mM) and inhibited by 1 mM ouabain. Black lines = control monolayers, grey lines = monolayers pretreated for 2.5 h with aldosterone (10^{-6} M) (see Table 1 for mean values).

whereas no significant effect was detectable at high Na concentrations (20 and 90 mM). The maximal absolute value of I_p induced by aldosterone (0.1 to $0.3 \mu\text{A}/\text{cm}^2$) represented only 1.4 to 4.3% of the $I_{p(\text{max})}$. The effect of aldosterone was abolished by the translation inhibitor cycloheximide added simultaneously with aldosterone at a concentration ($0.5 \mu\text{g}/\text{ml}$) that inhibits, in A6 cells, approximately 75% of the protein synthesis and >90% of the aldosterone-dependent increase in Na transport (Verrey *et al.*, 1987).

A6-C1 Cell Lines Expressing an Exogenous Na,K-ATPase $\alpha 1$ Subunit

A6-C1 cell lines stably expressing a transfected, cardiotoxic steroid-resistant Na,K-ATPase $\alpha 1$ subunit were generated to test whether the aldosterone-induced increase in apparent Na affinity (at low Na concentrations) could be due to an effect on the known Na pump population containing the $\alpha 1$ subunit or, alternatively, to the activation of an unknown subpopulation of pumps containing another α subunit isoform. For this purpose we chose $\alpha 1\text{TBM}$, which conferred an intermediate level of cardiotoxic steroid resistance to hybrid pumps (consisting of $\alpha 1\text{TBM}$ and an endogenous β subunit from *X. laevis*) compared with the endogenous Na,K-ATPase of A6-C1 cells (Jaisser *et al.*, 1992). Stable cell lines were obtained by ring cloning after selection in culture medium supplemented with $20 \mu\text{M}$ ouabain. These cell lines were

tested for the expression of $\alpha 1\text{TBM}$ by Western blotting and selected for their ability to form tight epithelia when cultured on filter supports. Figure 3 shows a Western blot of microsomal proteins from TBM-54 (toad bladder), A6-C1, and transfected, cardiotoxic steroid-resistant cell lines probed with an $\alpha 1\text{TBM}$ -specific antibody (see MATERIALS AND METHODS). Almost no cross-reactivity with the A6-C1 $\alpha 1$ subunit was detected (Figure 3, lanes 1–5). Lanes 6–9 demonstrate the expression of the $\alpha 1\text{TBM}$ subunit in the two cell lines that were obtained from independent trans-

Figure 3. Western blot showing the expression of $\alpha 1\text{TBM}$ in stably transfected A6-C1 cells. Microsomal proteins were prepared from epithelia of TBM-54, A6-C1, and transfected (1c and 3a) cells. Na butyrate pretreatment of the cells is indicated. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with an anti- $\alpha 1\text{TBM}$ antibody. A test of antibody specificity is shown in lanes 1–4, for which TBM-54 and A6-C1 proteins ($10 \mu\text{g}$ per lane) were mixed at different ratios (lane 1, TBM-54 only; lane 2, 50% each; lane 3, 10% TBM-54 and 90% A6-C1; lane 4, A6-C1 only). In lanes 5–9 ($20 \mu\text{g}$ protein per lane), the expression of the transfected $\alpha 1\text{TBM}$ is shown (lane 5, untransfected A6-C1; lanes 6 and 7, cell line 1c; lanes 8 and 9, cell line 3a).

fections. A sodium butyrate treatment increased the level of $\alpha 1$ TBM expression in cell line 1c (Figure 3, lanes 6 and 7), whereas it had no effect on cell line 3a (Figure 3, lanes 8 and 9).

To test whether the transfected $\alpha 1$ TBM subunit was functionally active, $I_{p[\max]}$ induced with 90 mM Na was inhibited by the addition of increasing concentrations of the cardiotonic steroid strophanthidin to the basolateral compartment. An example of dose-inhibition curves from the cell lines TBM-54, A6-C1 (untransfected), and 1c (transfected) is shown in Figure 4. The strophanthidin concentrations for half-maximal inhibition (K_i) were obtained by fitting curves corresponding to equations describing a single binding site (A6-C1) or a two independent-binding sites model (TBM-54 and 1c). The highest strophanthidin concentration applied was 250 μ M, and ouabain (2 mM) was used to reach 100% inhibition. The K_i values for strophanthidin were $82 \pm 3 \mu$ M ($n = 5$) for the TBM-54 and 177 ± 7 nM ($n = 11$) for the A6-C1 cell line and were not affected by aldosterone. To obtain a good fit to the experimental points from TBM-54 cells we had to use a model for two pump populations with different strophanthidin affinities. A minor population with a higher affinity ($K_i 1.1 \pm 0.2 \mu$ M) represented approximately 20% of the total binding sites in control epithelia and 10% in long-term aldosterone-treated epithelia (in which the total number of binding sites was increased by a factor of approximately two).

In the transfected, cardiotonic steroid-resistant cell lines the results clearly demonstrated the presence, besides the endogenous pumps, of a second Na pump population (see cell line 1c in Figure 4). The K_i values

of the hybrid pumps for strophanthidin ($58 \pm 9 \mu$ M, $n = 6$ for 1c; $62 \pm 14 \mu$ M, $n = 4$ for 3a) were close to that of the major pump population of TBM-54 cells. From these experiments we estimated the fraction of the I_p carried by hybrid pumps containing the $\alpha 1$ TBM subunit to be one-third in cell line 1c after Na butyrate and $\leq 10\%$ without pretreatment. In cell line 3a it amounted to 20% of the total current, independent of Na butyrate. The fact that these cardiotonic steroid-resistant I_p correlated with the relative expression of the $\alpha 1$ TBM subunit measured in the same cell lines by Western blotting (Figure 3) confirmed that this current was carried by the hybrid pumps containing the $\alpha 1$ TBM subunit.

Aldosterone also Acts on the Hybrid Pumps Containing the Transfected $\alpha 1$ TBM Subunit

We could now ask the question of whether aldosterone acts on pumps containing an $\alpha 1$ subunit by measuring its effect on the current carried by cardiotonic steroid-resistant hybrid pumps containing the $\alpha 1$ TBM subunit. Measurements of the transepithelial Na transport and of the total I_p (Table 2) in epithelia pretreated with butyrate indicated that this pretreatment did not prevent the action of aldosterone (2.5 h). Interestingly, in this series of experiments the effect of a short aldosterone treatment on the total I_p (at 5 mM Na) was larger than in the absence of butyrate (approximately 3-fold vs. 1.7-fold increase). Strophanthidin (50 μ M) was added to block the endogenous pump population such that the addition of Na would activate the I_p carried by hybrid pumps only (Figure 5, see also Fig-

Figure 4. Dose-dependence of the inhibition of the Na pump current (I_p) by strophanthidin. The I_p activated by 90 mM Na was measured across apically permeabilized epithelia of the cell lines A6-C1 (open circles), TBM-54 (closed circles), and 1c (expressing the $\alpha 1$ TBM subunit, Na butyrate pretreated; closed squares). Strophanthidin was added at increasing concentrations to the basolateral medium. The inhibition obtained with 2 mM ouabain was considered as maximal. Hyperbolic curves corresponding to one or two binding site(s) models were fitted to the experimental data (see RESULTS for mean K_i values).

Table 2. Short-term aldosterone (2.5 h; 10^{-6} M) increases I_p at low Na concentration (5 mM) carried by pumps containing the $\alpha 1$ TBM subunit in apically permeabilized epithelia of transfected A6-C1 cells

Cell line	Mean $I_p \pm SE$ ($\mu A/cm^2$) no strophanthidin			Mean $I_p \pm SE$ ($\mu A/cm^2$) + 50 μM strophanthidin basolateral		
	control	aldo	% increase	control	aldo	% increase
A6-C1	0.14 $\pm$ 0.01	0.37 $\pm$ 0.02	169 $\pm$ 8**	0.007 $\pm$ 0.002	0.006 $\pm$ 0.003	—
1c	0.087 $\pm$ 0.005	0.250 $\pm$ 0.015	193 $\pm$ 19**	0.029 $\pm$ 0.003	0.091 $\pm$ 0.007	228 $\pm$ 28**
3a	0.103 $\pm$ 0.007	0.253 $\pm$ 0.032	149 $\pm$ 37*	0.015 $\pm$ 0.001	0.044 $\pm$ 0.003	188 $\pm$ 30*

Monolayers were pretreated with butyrate. The I_p values represent means ($\pm$ SE from three to five paired experiments) of the current, inhibited within 1 min by 1 mM ouabain. Paired two-tailed t -test of test vs. control: * $P < 0.05$, ** $P < 0.01$.

ure 4). In contrast to untransfected A6-C1 cells, an I_p was measurable under these conditions in the transfected cell lines 1c and 3a. The pretreatment of the epithelia with aldosterone (2.5 h) increased by approximately 200% the strophanthidin-resistant current activated by 5 mM Na (Figure 5 and Table 2), similar to the effect measured in the absence of strophanthidin. This result showed that aldosterone acts by modifying the Na kinetics of pumps containing an $\alpha 1$ subunit.

Long-term Aldosterone Increases the $I_{p(max)}$

We have previously shown that during its late phase of action aldosterone induces an increase in the number of cell-surface pump sites measured by ouabain binding and by the immunoprecipitation of surface-labeled Na,K-ATPase molecules in A6-C1 cells (Beron and Verrey, 1994a). As shown in Table 3, the $I_{p(max)}$ measured in this study was increased by long-term aldosterone (4 days 10^{-6} M) to a similar extent, demonstrating that an increase in the maximal pumping capacity correlates with the increase in pump sites measured in the previous study. In conclusion, aldo-

sterone acts in two ways on Na pumps containing an $\alpha 1$ subunit. During its early phase of action it increases the apparent affinity for low Na concentrations of such Na pumps whereas in the long-term it also increases their total number and, consequently, the maximal I_p .

DISCUSSION

Regulation of the Endogenous Na Pumps by Aldosterone: Early Increase in Sodium Sensitivity and Late Increase in Pump Number

Sodium reabsorption across epithelial cells implies sequential transport steps across the apical and basolateral membranes such that the rates of apical Na influx through the channels and basolateral extrusion by the pumps must be regulated in parallel.

Several mechanisms may (partially) account for such a parallel regulation. First, constitutive kinetic properties of the active Na pumps (briefly mentioned in the INTRODUCTION) are at the origin of a steep increase in pump turnover rate (transport activity) in response to an increase in intracellular Na concentra-

Figure 5. Short-term aldosterone (2.5 h; 10^{-6} M) increases the I_p carried by hybrid pumps containing the $\alpha 1$ TBM subunit at low Na concentrations. Original recordings of the I_p measured in Na butyrate pretreated epithelia in the presence of 50 μM strophanthidin are shown. Currents were activated by 5 mM Na and inhibited by 1 mM ouabain at the indicated time points. (A) Untransfected A6-C1 cells. (B) cardiotoxic steroid-resistant cell line 1c. Black lines = control monolayers, grey lines = monolayers pretreated for 2.5 h with aldosterone (10^{-6} M) (see Table 2 for mean values).

Table 3. Long-term aldosterone (4 days; 10^{-6} M) increases $I_{p(max)}$ in epithelia of A6-C1 cells apically permeabilized with amphotericin B

Method	Mean $I_{p(max)} \pm SE$ ($\mu A/cm^2$)		n	% increase
	control	long-term aldo		
Curve fitting	4.5 ± 0.6	$8.7 \pm 1.0^*$	5	111 ± 37
90 mM Na	7.5 ± 0.5	$11.0 \pm 1.1^*$	5	47 ± 11

$I_{p(max)}$ was either derived from curve fitting on experimental points obtained as for Figure 1 (maximal Na concentration 40 mM) or directly measured at 90 mM Na as in Figure 4. The $I_{p(max)}$ values represent means $\pm SE$ from n paired experiments. Paired two-tailed *t*-test of test vs. control: **P* < 0.05.

tion. For instance, from the parameters derived by curve fitting (Hill coefficient = 2.4, see RESULTS) one can deduce that a doubling of the intracellular Na concentration produces, at low Na concentrations, an approximately fivefold increase in pump function. Second, a coordination of the Na transport function of the apical and the basolateral transporters (cross-talk) has been documented in several systems and might be indirectly mediated by effects of the Na fluxes on the cellular pH (in amphibian epithelia), Ca (feedback regulation of the Na channels), and/or vol (Schultz, 1981; Harvey *et al.*, 1988; Chuard and Durand, 1992; Frindt *et al.*, 1993; Silver *et al.*, 1993). Third, some hormones/factors may act in a coordinated manner at the level of the transporters of both membrane domains. This is the case with aldosterone in A6 cells. During its early phase of action it produces an increase in the open probability of apical Na channels (Kemeny *et al.*, 1992) and, as demonstrated in this study, it modulates the kinetics of the Na pump function. Besides these early concerted actions on the function of preexisting transport proteins, aldosterone is known to act, in A6 cells, on the biosynthesis of both the apical Na channel and the basolateral Na,K-ATPase (Verrey *et al.*, 1989; Verrey, 1995; Puoti *et al.*, 1994). The consecutive increase in Na pump number has been shown to take place only during the late phase of aldosterone action (Beron and Verrey, 1994a).

The most relevant kinetic characteristic of Na pumps is, in respect to Na transport activity, their Na sensitivity for the range of physiological intracellular Na concentrations. Indeed, this parameter (apparent Na affinity) gives information about the functional state of Na pumps (independent of the substrate effect) because it indicates at what rate pumps extrude Na from the cell at given intracellular Na concentrations. To measure this parameter, the intracellular Na concentration must either be controlled or measured simultaneously with the pump (or ATPase) activity. This should be done with minimal disruption of the

integrity of the epithelial and cellular structures, to preserve regulatory signals/mediators acting at the level of the Na pumps and that might otherwise be lost.

The method of permeabilizing the apical membrane of monolayers to monovalent ions with polyene antibiotics, for instance amphotericin B, fulfills these requirements (De Kruijff *et al.*, 1974). The very high conductance for monovalent ions produced selectively across the apical membrane allows for control of the intracellular monovalent cation concentrations and electrical potential without disrupting the basolateral or the paracellular barriers (Kirk *et al.*, 1980; Lewis and Wills, 1983; Palmer and Speez, 1986). The current generated by the Na pump, which is activated by Na and inhibited by ouabain, can then be monitored using a transepithelial voltage-clamp device (see MATERIALS AND METHODS AND RESULTS).

Using this technique, we have first generated Na activation curves by increasing the Na concentration stepwise and then fitting to the experimental points simple sigmoidal curves corresponding to a generalized Hill equation (De Weer, 1992; and refs. therein). The results of these experiments, which are essentially representative of the pump function at medium and high Na concentrations (upper part of the curve; see RESULTS), did not show any significant difference between control and short-term aldosterone-treated epithelia at the level of the maximal current ($I_{p(max)}$) or at the Na concentration required for half-maximal activation ($K_{0.5}$). However, the fact that a long aldosterone treatment increased the $I_{p(max)}$ represents a functional correlate to our previous observation that such a treatment increases the number of cell-surface pumps (Beron and Verrey, 1994a).

In contrast to the situation at medium and high Na concentrations, a short aldosterone treatment produced a clear increase in the level of the I_p when the effect was tested at low Na concentrations (Figure 2 and Table 1). A possible explanation for this result is that a short aldosterone treatment increases the apparent Na affinity of (a fraction of the) already active pumps. An alternative explanation is that aldosterone activates an otherwise silent subpopulation of pumps with a high affinity for Na that could contain another α subunit isoform. From the results mentioned above it is also clear that a simple sigmoidal curve fitted to the experimental points obtained in the low range of Na concentrations could not correctly describe the upper part of the Na activation curve. Close matches were obtained when curves corresponding to models for two pump populations with different apparent Na affinities and the same Hill coefficients were fitted to the data of Table 1. The fits indicated that the early effect of aldosterone, which represents a 1.7-fold increase in Na transport at physiological Na concentrations, could be mediated by the activation of a sub-

population of pumps with higher apparent Na affinity representing only a few percent of the total pumps. Because such a small fraction of the pumps would be required, it is not possible, based on our previous study on the Na,K-ATPase cell-surface expression, to conclude whether the activation corresponds to an *in situ* effect or is mediated by the appearance of new pump units at the cell surface (Beron and Verrey, 1994a); one has to keep in mind, however, the limitation of that curve-fitting approach. On the one hand, the precision and the number of the experimental points is limited and on the other hand, sigmoidal curves do not correspond to a realistic kinetic model for the function of the Na,K-ATPase (De Weer, 1992).

Aldosterone Effect Measured on an Exogenous $\alpha 1$ Subunit

The expression of Na,K-ATPase α subunits conferring (naturally or as a consequence of mutations) cardiotonic steroid resistance to host cells with sensitive endogenous pumps has been widely used as a means to study the function of exogenous α subunits while the endogenous pumps were blocked with ouabain (Kent *et al.*, 1987; Price and Lingrel, 1988; Canessa *et al.*, 1994). The expression of the $\alpha 1$ subunit of the toad *Bufo marinus* (Jaisser *et al.*, 1992), which was used in this study, has the advantage of conferring an intermediate level of cardiotonic steroid resistance to hybrid pumps containing an endogenous β subunit. Their function can be tested in the presence of ouabain (or other cardiotonic steroids) at a concentration that blocks endogenous pumps entirely while the hybrid pumps can subsequently be completely inhibited with high ouabain concentrations.

Because aldosterone (2.5 h) acted on pumps containing the transfected $\alpha 1$ subunit (Figure 5), we can deduce that the early effect of aldosterone observed in wild-type A6-C1 cells is independent of the regulation of the Na,K-ATPase $\alpha 1$ subunit gene and is most probably due to a change at the level of pumps containing the known $\alpha 1$ subunit and not to the activation of a subpopulation containing another α subunit isoform. On the basis of the present results, we cannot decide whether this effect on Na pumps containing an $\alpha 1$ subunit results from the modification of the kinetics of (a part of) the active pumps or from the activation of a subpopulation with different kinetics (also containing the $\alpha 1$ subunit).

The possibility of expressing cardiotonic steroid-resistant α subunits in A6-C1 cells now opens the possibility of studying structural requirements for regulatory functions in the context of a well differentiated and characterized epithelium.

Activation of Na Pumps by Aldosterone: Physiological Implications

The increase in the apparent Na affinity induced by a short aldosterone treatment might compensate for the increase in apical Na influx in such a way that it prevents an increase in intracellular Na. Such a stability of the intracellular Na after stimulation with aldosterone has been reported (Palmer and Speez, 1986; Bastl and Hayslett, 1992; and refs. therein). In that respect, it is also interesting to note that the time course of the aldosterone effect on the Na,K-ATPase measured as an increase in ouabain binding rate (Beron and Verrey, 1994b) as well as in I_p (our unpublished observation) parallels that of the increase in Na transport.

The increase in apparent affinity of the pumps for Na is expected to decrease the intracellular Na concentration, so it should increase the driving force for apical Na influx. Such an effect might play an important role for optimizing Na reabsorption in conditions of low luminal Na such as in states of Na deprivation. As yet, studies addressing the question of a putative aldosterone effect on the Na kinetics of the Na,K-ATPase have had negative results, in the case of the rabbit urinary bladder or the cortical collecting duct after long-term treatments (low Na diet) (Lewis and Wills, 1983; Welling *et al.*, 1993). In contrast, experiments performed on the toad bladder suggested the presence of an effect on the pump kinetics (Palmer and Speez, 1986). However, the fact that the apparent Na affinity of the mammalian Na,K-ATPases can be modulated has been documented in the cases of stimulation by insulin and adrenergic agonists in adipocytes and skeletal muscle and also in kidney proximal tubule (Aperia *et al.*, 1992; Féraile *et al.*, 1994; Lytton *et al.*, 1994). At present it is not clear whether the Na-independent early aldosterone effects (detected on rat cortical collecting duct Na,K-ATPase by measurements of Rb uptake and enzyme activity) are related to the kinetic changes observed in A6 cells (Barlet-Bas *et al.*, 1988; Fujii *et al.*, 1990).

The identity of the mechanism by which aldosterone acts on the apparent affinity of the Na,K-ATPase containing an $\alpha 1$ subunit remains unknown. The cycloheximide experiments show that protein synthesis must take place and suggest that it is mediated by the transcriptional induction/repression of a gene(s) by activated corticosteroid hormone receptor. The question of whether the mineralocorticoid and/or the glucocorticoid receptor is involved has not yet been addressed. Such a transcriptionally induced/repressed regulator could act directly or indirectly on Na pumps. It could change the Na kinetics of pumps or, alternatively, activate pumps with different kinetics by either noncovalently interacting with them or by covalently modifying them (Verrey, 1995).

In conclusion, short-term aldosterone produces, by a protein synthesis-dependent mechanism in A6-C1 cells, an increase in the apparent Na affinity of the Na pumps containing the $\alpha 1$ subunit, for physiological Na concentrations. This result shows that aldosterone exerts, besides its early effect on preexisting apical Na channels, a concerted early effect on the function of basolateral Na pumps, which is independent of the regulation of the Na,K-ATPase $\alpha 1$ subunit gene.

ACKNOWLEDGMENTS

The authors thank Bernard Rossier and Jean-Daniel Horisberger for stimulating discussions and comments on the manuscript, Ian Forster for reading the manuscript, Uschi Bolliger for cell culture work, Frédéric Jaisser, Hans-Peter Gaeggeler, and B. Rossier for kindly providing the $\alpha 1$ TBM cDNA, TBM-54 cells, and the $\alpha 1$ TBM peptide, and Christian Gasser for the artwork. This work was supported by grant 31-39449.93 from the Swiss National Science Foundation.

REFERENCES

- Ackerman, U., and Geering, K. (1990). Mutual dependence of Na, K-ATPase α - and β -subunit for correct posttranslational processing and intracellular transport. *FEBS Lett.* 269, 105-108.
- Aperia, A., Ibarra, F., Svensson, L.B., Klee, C., and Greengard, P. (1992). Calcineurin mediates α -adrenergic stimulation of Na^+ , K^+ -ATPase activity in renal tubule cells. *Proc. Natl. Acad. Sci. USA* 89, 7394-7397.
- Barlet-Bas, C., Khadoury, C., Marsy, S., and Doucet, A. (1988). Sodium-independent in vitro induction of Na^+ , K^+ -ATPase by aldosterone in renal target cells: permissive effect of triiodothyronine. *Proc. Natl. Acad. Sci. USA* 85, 1707-1711.
- Bastl, C.P., and Hayslett, J.P. (1992). The cellular action of aldosterone in target epithelia. *Kidney Int.* 42, 250-264.
- Beron, J., and Verrey, F. (1994a). Aldosterone induces early activation and late accumulation of Na-K-ATPase at surface of A6 cells. *Am. J. Physiol.* 266, C1278-C1290.
- Beron, J., and Verrey, F. (1994b). Aldosterone induces a delayed parallel increase in total and cell-surface Na^+ / K^+ -ATPase in A6 kidney cells. In: *The Sodium Pump*, ed. E. Bamberg and W. Schoner, Darmstadt, Germany: Steinkopff, 238-241.
- Broillet, M.C., and Horisberger, J.D. (1991). Basolateral membrane potassium conductance of A6 cells. *J. Membr. Biol.* 124, 1-12.
- Canessa, C., Jaisser, F., Horisberger, J.D., and Rossier, B.C. (1994). Structure-function relationship of Na, K-ATPase: the digitalis receptor. In: *Cell Biology and Membrane Transport Processes*, ed. M. Caplan, San Diego, CA: Academic Press, 71-85.
- Chuard, F., and Durand, J. (1992). Coupling between the intracellular pH and the active transport of sodium in an epithelial cell line from *Xenopus laevis*. *Comp. Biochem. Physiol.* 102, 7-14.
- De Kruijff, B., Gerritsen, W.J., Oelermans, A., Demel, R.A., and van Denen, L.L.M. (1974). Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by the polyene antibiotics. *Biochim. Biophys. Acta* 339, 30-43.
- De Weer, P. (1992). Cellular sodium-potassium transport. In: *The Kidney: Physiology and Pathophysiology*, 2nd ed., ed. D.W. Seldin and G. Giebisch, New York: Raven, 93-112.
- Férraille, E., Carranza, M.L., Rousselot, M., and Favre, H. (1994). Insulin enhances sodium sensitivity of Na-K-ATPase in isolated rat proximal convoluted tubule. *Am. J. Physiol.* 267, F55-F62.
- Forbush, B. (1983). Cardiotonic steroid binding to Na, K-ATPase. *Curr. Top. Membr. Transp.* 19, 167-201.
- Frindt, G., Silver, R.B., Windhager, E.E., and Palmer, L.G. (1993). Feedback regulation of Na channels in rat CCT. II: Effects of inhibition of Na entry. *Am. J. Physiol.* 264, F565-F574.
- Fujii, Y., Takemoto, F., and Katz, A.I. (1990). Early effects of aldosterone on Na-K pump in rat cortical collecting tubules. *Am. J. Physiol.* 259, F40-F45.
- Garty, H. (1994). Molecular properties of epithelial, amiloride-blockable Na^+ channels. *FASEB J.* 8, 522-528.
- Granitzer, M., Leal, T., Nagel, W., and Crabbé, J. (1991). Apical and basolateral conductance in cultured A6 cells. *Pflügers Arch.* 417, 463-468.
- Handler, J.S., Steele, R.E., Sahib, M.K., Wade, J.B., Preston, A.S., Lawson, N.L., and Johnson, J.P. (1979). Toad urinary bladder epithelial cells in culture: maintenance of epithelial structure, sodium transport, and response to hormones. *Proc. Natl. Acad. Sci. USA* 76, 4151-4155.
- Harvey, B.J., Thomas, S.R., and Ehrenfeld, J. (1988). Intracellular pH controls cell membrane Na^+ and K^+ conductances and transport in frog skin epithelium. *J. Gen. Physiol.* 92, 767-791.
- Jaisser, F., Canessa, C.M., Horisberger, J.D., and Rossier, B.C. (1992). Primary sequence and functional expression of a novel ouabain-resistant Na, K-ATPase: the β -subunit modulates potassium activation of the Na, K-pump. *J. Biol. Chem.* 267, 16895-16903.
- Kemendy, A.E., Kleyman, T.R., and Eaton, D.C. (1992). Aldosterone alters the open probability of amiloride-blockable sodium channels in A6 epithelia. *Am. J. Physiol.* 263, C825-C837.
- Kent, R.B., Emanuel, J.R., Neriah, Y.B., Levenson, R., and Housman, D.E. (1987). Ouabain resistance conferred by expression of the cDNA for a murine Na^+ , K^+ -ATPase α -subunit. *Science* 237, 901-903.
- Kirk, K.L., Halm, D.R., and Dawson, D.C. (1980). Active sodium transport by turtle colon via an electrogenic Na-K exchange. *Nature* 287, 237-239.
- Kleyman, T.R., Cragoe, E.J., and Kraehenbuhl, J.P. (1989). The cellular pool of Na^+ channels in the amphibian cell line A6 is not altered by mineralocorticoids. *J. Biol. Chem.* 264, 11995-12000.
- Lewis, S.A., and Wills, N.K. (1983). Apical membrane permeability and kinetic properties of the sodium pump in rabbit urinary bladder. *J. Physiol.* 341, 169-184.
- Lytton, J., Lin, J., DiAntonio, L., Brodsky, J., McGeoch, J., McGill, D., and Guidotti, G. (1994). Regulation of the Na^+ , K^+ -pump by insulin. In: *The Sodium Pump*, ed. E. Bamberg and W. Schoner, Darmstadt, Germany: Steinkopff, 670-681.
- Paccolat, M.P., Geering, K., Gaeggeler, H.P., and Rossier, B.C. (1987). Aldosterone regulation of Na^+ transport and Na^+ - K^+ -ATPase in A6 cells: role of growth conditions. *Am. J. Physiol.* 252, C468-C476.
- Palmer, L.G., and Speez, N. (1986). Stimulation of apical Na permeability and basolateral Na pump of toad urinary bladder by aldosterone. *Am. J. Physiol.* 250, F273-F281.
- Pácha, J., Frindt, G., Antonian, L., Silver, R.B., and Palmer, L.G. (1993). Regulation of Na channels of the rat cortical collecting tubule by aldosterone. *J. Gen. Physiol.* 102, 25-42.
- Pellanda, A.M., Gaeggeler, H.P., Horisberger, J.D., and Rossier, B.C. (1992). Sodium-independent effect of aldosterone on initial rate of ouabain binding in A6 cells. *Am. J. Physiol.* 262, C899-C906.

- Posnett, D.N., and Tam, J.P. (1989). Multiple antigenic peptide method for producing antipeptide site-specific antibodies. *Methods Enzymol.* 178, 739–746.
- Price, E.M., and Lingrel, J.B. (1988). Structure-function relationships in the Na, K-ATPase alpha subunit: site-directed mutagenesis of glutamin-111 to arginine and asparagine-122 to aspartic acid generates an ouabain-resistant enzyme. *Biochemistry* 27, 8400–8408.
- Puoti, A., Canessa, C., and Rossier, B.C. (1994). Cloning and functional analysis of the amiloride-sensitive epithelial sodium channel of *X. laevis*. *Experientia* 50, A41 (abstract).
- Rossier, B.C., and Palmer, L.G. (1992). Mechanisms of aldosterone action on sodium and potassium transport. In: *The Kidney: Physiology and Pathophysiology*, 2nd ed., ed. D.W. Seldin and G. Giebisch, New York: Raven, 1373–1409.
- Schultz, S.G. (1981). Homocellular regulatory mechanisms in sodium transporting epithelia: avoidance of extinction by “flush-through.” *Am. J. Physiol.* 241, F579-F590.
- Silver, R.B., Frindt, G., Windhager, E.E., and Palmer, L.G. (1993). Feedback regulation of Na channels in rat CCT. 1. Effects of inhibition of Na pump. *Am. J. Physiol.* 264, F557-F564.
- Truscello, A., Geering, K., Gaeggeler, H.P., and Rossier, B.C. (1983). Effects of butyrate on histone deacetylation and aldosterone-dependent Na⁺ transport in the toad bladder. *J. Biol. Chem.* 258, 3388–3395.
- Verrey, F. (1990). Regulation of gene expression by aldosterone in tight epithelia. *Semin. Nephrol.* 10, 410–420.
- Verrey, F. (1994). Antidiuretic hormone action in A6 cells: effect on apical Cl and Na conductances and synergism with aldosterone for NaCl reabsorption. *J. Membr. Biol.* 138, 65–76.
- Verrey, F. (1995). Transcriptional control of sodium transport in tight epithelia by adrenal steroids. *J. Membr. Biol.* (in press).
- Verrey, F., Kraehenbuhl, J.P., and Rossier, B.C. (1989). Aldosterone induces a rapid increase in the rate of Na, K-ATPase gene transcription in cultured kidney cells. *Mol. Endocrinol.* 3, 1369–1376.
- Verrey, F., Schaerer, E., Zoerkler, P., Paccolat, M.P., Geering, K., Kraehenbuhl, J.P., and Rossier, B.C. (1987). Regulation by aldosterone of Na⁺, K⁺-ATPase mRNAs, protein synthesis, and sodium transport in cultured kidney cells. *J. Cell Biol.* 104, 1231–1237.
- Welling, P.A., Caplan, M., Sutters, M., and Giebisch, G. (1993). Aldosterone-mediated Na/K-ATPase expression is alpha(1) isoform specific in the renal cortical collecting duct. *J. Biol. Chem.* 268, 23469–23476.