Direct Action of Aldosterone on Transmembrane 22Na Efflux from Arterial Smooth Muscle
Rapid and Delayed Effects

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SUMMARY Acute subcutaneous (s.c.) administration of aldosterone increases ex vivo 22Na efflux from rat tail artery smooth muscle, which appears to be due to a specific action on mineralocorticoid receptors. Indeed, this effect is blocked by the antimineralocorticoid compounds RU 28318 [17β-hydroxy-3-oxo, 7α-propyl(17α)-pregn 4-ene, 21 potassium carboxylate] and spironolactone. The specific glucocorticoid receptor agonist RU 26988 [11β,17β-dihydroxy-17-(1-propynyl) androsta-1,4,6 trien-3-one] does not modify 22Na efflux. We show here that aldosterone has, at physiological concentrations, a mineralocorticoid specific stimulating effect on passive and sodium pump dependent transmembrane movements of sodium from the rat tail artery smooth muscle. Aldosterone exerts two types of action on sodium transport: 1) a delayed stimulation of ouabain-dependent 22Na efflux and ouabain-independent 22Na efflux, which are completely blocked by actinomycin D; and 2) a very rapid increase of passive 22Na efflux, which is insensitive to actinomycin D and therefore does not seem to depend on transcription of genomic information. (Hypertension 6: 425-430, 1984)

KEY WORDS vascular smooth muscle 22Na transmembrane movements of Na mineralocorticoid antimineralocorticoids RU 28318 spironolactone glucocorticoids genoma aldosterone

In the long term, regulation of salt metabolism depends on aldosterone effects on Na+, K+, H+, and H2O transport by the renal tubules. Furthermore, it has been shown that aldosterone modifies other epithelial transports, inducing a positive sodium balance. The overall action of the hormone may be important in the regulation of blood pressure, an excess of mineralocorticoid hormones being associated with hypertension in primary aldosteronism. It has been shown that, besides its effects on transepithelial transport, the chronic in vivo administration of aldosterone modifies transmembrane ionic fluxes in vascular smooth muscle.5 Recently,6 using a technique of exchange of Na+ by Li+, Friedman observed that, in vitro, aldosterone reduces free cell sodium in vascular smooth muscle cells of the rat tail artery. Furthermore, no change in membrane permeability was found. Garwitz and Jones3 consequently suggested that aldosterone may enhance net Na+ transport through the stimulation of the sodium pump.

The aim of the present work was to study the mechanism of action of aldosterone on 22Na flux and to determine if antialdosterone compounds may block mineralocorticoid effects at the level of the vascular smooth muscle. The results presented in this paper indicate that aldosterone had a direct stimulating action on ouabain-dependent and ouabain-independent 22Na efflux, both effects being blocked by antimineralocorticoid compounds.

METHODS

Animals

The experiments were performed on 200 g male Sprague Dawley rats that had been adrenalectomized by the supplier (IFCA-Credo, Les Oncins, France), and were maintained on 0.9% NaCl in water until they were studied, which was more than 7 days after adrenalectomy.

22Na-Efflux Measurements

The method used for the study of 22Na effluxes from the rat tail artery has been published elsewhere.7 In
summary, following excision and dissection, the rat tail artery was opened longitudinally and cut into two sections 1 cm long. One section was exposed to ouabain, and the other used for the total $^{22}\text{Na}$ efflux measurement. The tissues were loaded for 90 minutes at 37°C, with $^{22}\text{NaCl}$ (Radiochemical Centre, Amersham, Bucks, England) in physiological saline having the following composition (mM): Na$^+$ 136.9, K$^+$ 5.9, Ca$^{2+}$ 2.5, Mg$^{2+}$ 1.2, Cl$^-$ 133.5, H$_2$PO$_4^-$ 1.2, HCO$_3^-$ 15.5, and glucose 11.5, gassed with 95% O$_2$ and 5% CO$_2$, pH 7.3. The $^{22}\text{Na}$ efflux from the strip was studied using a continuous flow technique by superfusion. Before desaturation, the strips were rapidly washed in cold (4°C) physiologic solution for 15 minutes to eliminate excess radioactivity on the surface of the strip and in the extracellular space. Thereafter, the flow was adjusted to 2 ml/min, and the entire effluent was collected at 1-minute intervals. The radioactivity left in the tissue at the end of the experiment, as well as the activity of the washout tubes, was counted in a γ well counter. A desaturation curve was obtained by adding in reverse order the washout curve to the radioactivity remaining in the tissue. Some strips were exposed to 1 mM ouabain during the last 10 minutes of the initial washout period at 4°C, as well as during all the desaturation period performed as usual at 37°C. Under these conditions, the glycoside blocked the sodium pump completely. Consequently, it was possible to evaluate the ouabain-dependent (sodium pump) and ouabain-independent fractions of $^{22}\text{Na}$ efflux. Results are expressed as rate coefficient (min$^{-1}$), which is calculated by the expression:

$$r = A_1 - A_2 / (A_1 + A_2/2)(t_f - t_i)$$

where $r$ is the rate coefficient; $A_1$ and $A_2$ are two points in the desaturation curve at time $t_i$ and $t_f$. The effects of the drugs on $^{22}\text{Na}$ efflux rates were evaluated at the initial 12 minutes of washout. This part of the $^{22}\text{Na}$ desaturation curve is specifically dependent on transmembrane sodium movements from smooth muscle cells.

**Experimental Protocol**

For the ex vivo experiments, the animals were given subcutaneous injections of aldosterone dissolved in a 2.5% ethanol solution and killed by a blow on the head at different times. Control adrenalectomized animals were injected with the solvent. During the loading with $^{22}\text{Na}$ and during the washout, the arteries were studied more than 105 minutes after sacrifice, with the vessels exposed to the physiological saline solution in the absence of drugs. Antimineralocorticoid compounds (suspended in a carboxymethylcellulose 0.25% solution in H$_2$O with 0.2% polysorbate 80, in a total volume of 5 ml/kg) were administered, by gavage, 1 hour before the subcutaneous injection of aldosterone. Control adrenalectomized animals were treated with the solvent. In some experiments, the rats were pretreated 1 hour before the subcutaneous administration of aldosterone with 50 μg/kg of actinomycin D® dissolved in a 0.04% ethanol solution.

The in vitro experiments were done by exposing the rat tail artery to aldosterone (dissolved in 0.9% NaCl). The effluxes were measured either 15 minutes or 120 minutes after exposure to the mineralocorticoid. In some experiments, the antimineralocorticoid RU 28318 (dissolved in 0.9% NaCl) was added to the media 1 hour before the addition of aldosterone when the effluxes were measured 2 hours later, and 165 minutes before the mineralocorticoid when the strips were exposed to aldosterone for 15 minutes. Thus, the strips were always in the presence of the antimineralocorticoid for 120 minutes. Some strips were exposed to 40 μg/ml of actinomycin D® (dissolved in a 0.04% ethanol solution) 1 hour before the addition of aldosterone to the media.

**Drugs Used**

Aldosterone, spironolactone, RU 28318, and RU 26988 were synthetized by Roussel-Uclaf (Romainville, France). Ouabain octahydrate was purchased.
from Sigma Chemical Company (St Louis, Missouri). All salts were reagent grade.

Statistical Analysis

Results are presented as the means ± standard error of the mean. Significance was determined using the Dunnett test.

Results

Ex Vivo Effects of Aldosterone

Ionic flux experiments on the rat tail artery have revealed that aldosterone has an appreciable effect on transmembrane $^{22}$Na efflux. When injected subcutaneously in adrenalectomized rats, the mineralocorticoid increased the total $^{22}$Na efflux, measured ex vivo. Experiments presented in Figure 1 show that aldosterone (10 μg/kg) had a very rapid initial effect on the ouabain-insensitive $^{22}$Na efflux, which started as early as 15 minutes after the injection, and was followed by a secondary rise in efflux that appeared to attain a plateau 4 hours after the mineralocorticoid administration. Furthermore, under these conditions aldosterone increased the ouabain-dependent $^{22}$Na efflux; the initial effect was obtained after 1 hour and reached a plateau 3 hours later. The late increase in ouabain-insensitive $^{22}$Na efflux, as well as all the stimulating effects of aldosterone on the ouabain-dependent (sodium pump) efflux, were completely blocked by inhibition of protein synthesis with actinomycin D. The early increase in ouabain-independent $^{22}$Na efflux was not modified. Aldosterone appeared to have a dose-dependent effect on $^{22}$Na efflux, measured 2 hours after injection, with an ED$_{50}$ of 2 μg/kg (Figure 2). The effects of aldosterone injections appeared to be due entirely to an action of the hormone on mineralocorticoid receptors, since the specific mineralocorticoid antagonists RU 28318 (Figure 2) and spironolactone (Figure 3) blocked dose-dependently the hormone effects on $^{22}$Na effluxes. Nevertheless, the ouabain-dependent efflux (sodium pump) was more sensitive to the antimineralocorticoid action than the ouabain-insensitive $^{22}$Na efflux. Moreover, the subcutaneous injection of the specific glucocorticoid receptor agonist RU 26988 (11β, 17β-dihydroxy-17-[1-propynyl] androesta-1, 4,6 trien-3-one), had at the supramaximal dose of 5 mg/kg at which it occupied glucocorticoid-binding sites completely, had negligible effects on $^{22}$Na efflux (Figure 4). This suggests that these effects of aldosterone are not mediated by glucocorticoid receptors.

In Vitro Effects of Aldosterone

The aldosterone-induced modifications of $^{22}$Na effluxes appeared to be partly due to a direct action on arterial smooth muscle cells, as shown by the results of in vitro experiments (Figure 5). Under these conditions, the mineralocorticoid had the same effects on

FIGURE 2. In vivo effect of the antimineralocorticoid RU 28318 on the dose-$^{22}$Na efflux curves of aldosterone. • = total $^{22}$Na efflux; ■ = ouabain-insensitive $^{22}$Na efflux; ▲ = ouabain-dependent $^{22}$Na efflux; ○△ = the same after pretreating the rats with RU 28318. n = 10 rats. *p < 0.05. **p < 0.01.

FIGURE 3. In vivo effect of spironolactone on the dose-$^{22}$Na efflux curves of aldosterone. • = total $^{22}$Na efflux; ■ = ouabain-insensitive $^{22}$Na efflux; ▲ = ouabain-dependent $^{22}$Na efflux; ○△ = the same after pretreating the rats with spironolactone. n = 10 rats. *p < 0.05. **p < 0.01.
ouabain-dependent $^{22}$Na efflux as previously shown after in vivo administration, namely, a late increase giving rise to a plateau phase obtained after 3 hours. This effect is blocked by actinomycin D. On the other hand, in vitro exposure to aldosterone does not induce any change in ouabain-insensitive $^{22}$Na efflux. Under these conditions, only the initial rapid action was observed, starting 15 minutes after exposure of the mineralocorticoid on ouabain-insensitive $^{22}$Na efflux; the effects of actinomycin D on passive efflux being negligible. Measured 120 minutes after exposure to aldosterone (Figure 6), the antimineralocorticoid RU 28318 blocked the increase in ouabain-independent and ouabain-dependent $^{22}$Na efflux, induced by the mineralocorticoid.

The mechanism of the early and late effects of aldosterone on the ouabain-independent $^{22}$Na efflux may be similar, since the dose-response curves and the ED$_{50}$ (1 nM) were not different when measured after 15 and 120 minutes of exposure to the mineralocorticoid (Figure 7). Furthermore, the curves representing the blocking action of the antimineralocorticoid RU 28318 on the early and late passive effects of aldosterone were superimposable (Figure 8).

**Discussion**

The results of this work indicate that aldosterone increases, at doses that determine a renal effect in vivo, and at in vitro concentrations compatible with a physiological effect, both ouabain-independent and Na pump-dependent $^{22}$Na effluxes. These aldosterone effects appear to be mineralocorticoid-specific since they are blocked dose-dependently by aldosterone antagonists. Furthermore, the specific glucocorticoid receptor agonist RU 26988 has negligible effects at doses that saturate glucocorticoid receptors. The rat tail artery contains mineralocorticoid as well as glucocorticoid receptors (M.M. Bouton, unpublished data), agreeing with observations in rabbit aorta, femoral and carotid arteries, as well as in lamb and rabbit brain.
small arteries and in isolated smooth muscle cells.

Our results partly confirm Friedman's findings obtained by the technique of exchange of Na+ by Li+. Under these conditions he only observed an increase of the sodium pump activity in the rat tail artery with 3 hours of in vitro exposure to aldosterone, but no change in passive permeability to sodium. There is no obvious explanation for the lack of effect of the mineralocorticoid on passive sodium permeability unless this response varies in different experimental conditions. Indeed, we used adrenalectomized rats as opposed to Friedman who worked with no excised animals. Preliminary findings from our laboratory have shown that corticoids have a tonic physiological effect on transmembrane sodium fluxes, as basal total Na+ efflux from the rat tail artery is greatly reduced in adrenalectomized rats.

It has been repeatedly shown that the chronic administration of DOCA or aldosterone increases passive and pump-dependent sodium transport in vascular smooth muscle, but under these conditions of long-term in vivo administration, corticoid effects on membrane permeability may be due to direct or indirect effects (secondary hormonal release).

Aldosterone administered acutely in vivo or in vitro activates both passive and sodium-pump-mediated transport in the kidney and other aldosterone-sensitive tissues, after a delay of more than 1 hour. Since these effects are blocked by transcription and translation inhibitors, it has been suggested that they are mediated by an aldosterone-induced protein, synthesized by a mechanism triggered by the occupation of cytosolic and nuclear receptors.

Our results indicate that aldosterone has a direct and mineralocorticoid-specific action on the ouabain-dependent Na+ efflux from rat tail artery smooth muscle, which appears to be identical after in vitro exposure or in vivo administration of the hormone. In both experimental conditions, aldosterone-stimulating effects on the sodium pump appear after a long delay and are suppressed by actinomycin D. This suggests that this mineralocorticoid action follows the usually proposed mechanism involving the transcription of genomic information.

The in vivo effects of aldosterone on the ouabain-dependent Na+ efflux appear to be due to the addition of two consecutive effects: 1) the initial rapid stimulation, starting as early as 15 minutes after the administration of the hormone and attaining a plateau at 60 minutes; and 2) a late rise starting after 60 minutes, reaching a new plateau at 240 to 300 minutes.
The initial and late stimulating actions of aldosterone on passive $^{22}\text{Na}$ efflux are mineralocorticoid-specific, but only the early rapid effect appears to be due to a direct action of the hormone on vascular smooth muscle. Indeed, the late increase in passive (ouabain-insensitive) $^{22}\text{Na}$ efflux does not exist after in vitro exposure to aldosterone, thus suggesting that this component is secondary to the action of an unknown hormonal factor.

The early (direct) effect of the mineralocorticoid has a rapid onset — less than 15 minutes — hardly compatible with the time necessary for the activation of the target cell genome. Indeed, this action of aldosterone is not blocked by actinomycin D.

It has been observed that other steroid hormones may affect neural function with a delay that precludes the mediation of a DNA transcription step. For example, the firing of single cells in the tuberal hypothalamus of the rat appears within the 10 seconds that follow cortisol application. The action of steroids on membrane potential and transmembrane ionic transfer could result from a direct interaction at the level of the cell surface, as illustrated by the progesterone-induced meiotic division in Xenopus laevis oocytes. The membrane actions of steroids may result from either: 1) surface action, 2) selective interaction of steroid molecules with membranes, 3) steroid binding to membrane receptors, or 4) changes in function secondary to steroid interaction with classic cytosolic and nuclear receptors (D. Duval, unpublished data).

The early (15 minutes) in vitro effects of aldosterone observed in this work may be due to the action of the mineralocorticoid on the same receptors that are responsible for the late (120 minutes) in vitro stimulation of ouabain-independent $^{22}\text{Na}$ efflux. Indeed, early and late dose-response curves to aldosterone and the anti-mineralocorticoid RU 28318 appear to be very similar (Figures 7 and 8), suggesting that the receptors involved in the early (nongenomic) and late (genomic) responses have similar pharmacologic properties.

The direct and indirect effects of aldosterone on the transmembrane Na transport in arterial smooth muscle may be important for the regulation of the concentration of free intracellular sodium. It may be suggested that, as a consequence of this effect, the mineralocorticoid hormone participates in the physiologic modulation of vascular tonus and blood pressure. Nevertheless, in vitro studies performed on rat tail artery and aorta and rabbit aorta have failed to confirm this hypothesis. Furthermore, it has been found that deoxycorticosterone, but not aldosterone (which is inactive), reduces the contractile response of the rat tail artery to phenylephrine and other agonists. Further experiments are needed to ascertain the in vivo and in vitro effects of aldosterone on smooth muscle contraction.

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