Hyperaldosteronemia in Rabbits Inhibits the Cardiac Sarcolemmal Na\(^+\)-K\(^+\) Pump

Anastasia S. Mihailidou, Henning Bundgaard, Mahidi Mardini, Peter S. Hansen, Keld Kjeldsen, Helge H. Rasmussen

Abstract—Aldosterone upregulates the Na\(^+\)-K\(^+\) pump in kidney and colon, classical target organs for the hormone. An effect on pump function in the heart is not firmly established. Because the myocardium contains mineralocorticoid receptors, we examined whether aldosterone has an effect on Na\(^+\)-K\(^+\) pump function in cardiac myocytes. Myocytes were isolated from rabbits given aldosterone via osmotic minipumps and from controls. Electrogenic Na\(^+\)-K\(^+\) pump current, arising from the 3:2 Na\(^+\):K\(^+\) exchange ratio, was measured in single myocytes using the whole-cell patch clamp technique. Treatment with aldosterone induced a decrease in pump current measured when myocytes were dialyzed with patch pipette solution containing Na\(^+\) in a concentration of 10 mmol/L, whereas there was no effect measured when the solution contained 80 mmol/L Na\(^+\). Aldosterone had no effect on myocardial Na\(^+\)-K\(^+\) pump concentration evaluated by vanadate-facilitated \([\text{H}]\text{ouabain binding or by K}^+-\text{dependent paranitrophenylphosphatase activity in crude homogenates. Aldosterone induced an increase in intracellular Na}^+\text{ activity. The aldosterone-induced decrease in pump current and increased intracellular Na}^+\text{ were prevented by cotreatment with the mineralocorticoid receptor antagonist spironolactone. Our results indicate that hyperaldosteronemia decreases the apparent Na}^+\text{ affinity of the Na}^+\text{-K}^+\text{ pump, whereas it has no effect on maximal pump capacity.} (\text{Circ Res. 2000;86:37-42.})

Key Words: cardiac ■ mineralocorticoid receptor ■ spironolactone ■ ouabain binding ■ sodium

It is widely accepted that aldosterone increases abundance and activity of the Na\(^+\)-K\(^+\)-pump in kidney and colon. These organs are considered classical targets for aldosterone. Because the hormone can also bind with high affinity in the heart, effects on the cardiac sarcolemmal Na\(^+\)-K\(^+\) pump have been examined. In vitro exposure of cultured rat cardiac myocytes to physiologically relevant nanomolar concentrations of aldosterone increases the abundance of mRNA for the catalytic \(\alpha_1\) subunit of the Na\(^+\)-K\(^+\) pump, and when myocytes are exposed to micromolar concentrations, an increase in expression of the corresponding protein isoform can be demonstrated.

The effects of aldosterone have also been examined in vivo. In one study, rats were given aldosterone via osmotic minipumps at a dose that caused a \(\approx\)5-fold increase in serum levels. There was no change in mRNA levels for Na\(^+\)-K\(^+\) pump subunits in the heart after 1, 3, or 15 days of treatment, and the authors concluded that the myocardial Na\(^+\)-K\(^+\) pump is not regulated by aldosterone. In another study, guinea pigs were given aldosterone for 90 days via osmotic minipumps at a dose that produced a \(\approx\)2-fold increase in serum levels. Northern and Western blot analysis showed that aldosterone induced a substantial increase in mRNA and protein levels of the \(\alpha_2\) subunit, whereas there was no effect on the \(\alpha_1\) subunit. The authors suggested that the increase in the \(\alpha_2\) isoform would cause an increase in pump activity and, hence, a decrease in the intracellular Na\(^+\) concentration. However, neither pump activity nor intracellular Na\(^+\) levels were measured.

If Na\(^+\)-K\(^+\) pump gene expression is assumed to reflect activity, the previous studies suggest that aldosterone either has no effect or that it induces an increase in myocardial Na\(^+\)-K\(^+\) pump activity. However, pump function was not directly examined in any of these studies. In the present study, the effect of aldosterone on myocardial Na\(^+\)-K\(^+\) pump function was examined. Aldosterone was administered to rabbits via implanted osmotic minipumps to achieve increases in plasma levels similar to those encountered in human hyperaldosteronemia. We measured Na\(^+\)-K\(^+\) pump current \((I_p)\) in isolated ventricular myocytes using the whole-cell patch-clamp technique. Treatment with aldosterone induced a decrease in \(I_p\) measured when the intracellular Na\(^+\) concentration was set near physiological levels. However, there was no effect of treatment when Na\(^+\) was at a level expected to nearly saturate intracellular pump sites, suggesting that aldosterone has no effect on maximal pump capacity.
This conclusion was supported by the absence of an effect of aldosterone on vanadate-facilitated [3H]ouabain binding capacity in intact samples and K+-dependent paranitrophephosphatase (pNPPase) activity in crude myocardial homogenates. Taken together, our results indicate that hyperaldosteronemia induces a functionally significant decrease in myocardial Na+-K+ pump activity but has no effect on the number of pump units.

Materials and Methods
A total of 71 male New Zealand White rabbits weighing 2.5 to 3.0 kg were used. They were maintained on standard chow and had free access to tap water. In vivo interventions were followed by in vitro studies of cells or tissues. To administer aldosterone or spironolactone, we implanted osmotic minipumps (Alza) subcutaneously in the interscapular region under a general anesthetic of 2% halothane with 2 parts nitrous oxide and 1 part oxygen. Aldosterone and spironolactone were dissolved in a stock solution of ethanol and diluted in 0.9% sterile saline. Control rabbits were infused with the ethanol vehicle only. When treatment protocols were completed, we anesthetized rabbits with intramuscular ketamine (50 mg kg−1) and xylazine hydrochloride (20 mg/kg) and excised the heart. Experimental protocols were approved by the institutional ethics committee at Royal North Shore Hospital, Sydney, and at Rigshospitalet, Copenhagen, and were conducted in accord with Danish Ministry of Justice legislation.

Single myocytes from either ventricle were isolated and voltage clamped with wide-tipped (4- to 5-μm) patch pipettes with resistances of 0.9 to 1.1 MΩ, as described previously.10 For measurement of Ii, myocytes were superfused with Ca2+-containing modified Tyrode’s solution, as described previously.10 This solution was used while the whole-cell configuration was established and the membrane capacitance was measured. The superfusate was then changed to one that was identical except that it was nominally Ca2+-free and contained 0.2 mmol/L CaCl2 and 2 mmol/L BaCl2. Ii was identified in myocytes voltage clamped at −40 mV as the shift in holding current induced by 100 μmol/L ouabain. Currents are normalized for membrane capacitance. Details of the experimental setup and of the experimental protocols used to measure membrane capacitance and Ii have been described previously.10,11 Vanadate-facilitated [3H]ouabain binding to intact left ventricular samples of 2 to 4 mg (wet weight) was performed as described in detail for rat skeletal muscle.12 K+-dependent pNPPase activity was determined in crude homogenates (10 mg tissue/mL), as described previously for rat myocardium.13 Tissue K+ content was measured in samples of ~25 mg wet weight by flame photometry using lithium as an internal standard. All measurements were made in duplicate. Details have been described.14 Intracellular Na+ activity (aNa) was measured in intact isolated right ventricular papillary muscles with Na+-sensitive microelectrodes as described previously.10,11

Reagents and Chemicals
Aldosterone, spironolactone, ouabain, dihydroouabain (DHO), and pNPP were purchased from Sigma. Tetramethyl-ammonium chloride was of purum grade and was purchased from Fluka. [3H]Ouabain was from Amersham International. Vanadate was purchased from Merck. Chemicals used for the K+-dependent pNPPase activity, [3H]Ouabain binding, and tissue K+ content experiments were purchased from Bie and Bernten. All other chemicals were purchased from BDH. All chemicals were of analytical grade.

Statistical Analysis
Results are expressed as mean±SE. Statistical comparisons were made by both paired and unpaired Student t test and 1-way ANOVA followed by a Tukey test. Differences were regarded as statistically significant when P<0.05. An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results
A previous study on the renal effects of aldosterone in rabbits used a dose of 50 μg/kg body weight per day for 7 days.4 To determine an appropriate dose that would produce a clinically relevant increase in plasma aldosterone levels in our rabbits, we administered aldosterone in doses of 30, 50, or 100 μg/kg body weight per day via osmotic minipumps for 7 days. Blood was collected for plasma concentrations of aldosterone at the time of implantation of minipumps and immediately before the rabbits were euthanized. The relationship between the infused dose and levels of plasma aldosterone is shown in Figure 1. The dose of 50 μg/kg body weight per day used in the previous study4 induced an increase in aldosterone levels similar to that seen in hyperaldosteronemic states and was therefore adopted for our study. Unless specified otherwise, this dose infused for 7 days was used throughout the study.

Effect of Aldosterone on Serum and Tissue K+
Infusion of 50 μg/kg body weight per day aldosterone for 7 days produced a decrease in serum K+ from 4.8±0.1 to 3.6±0.2 mmol/L. The difference was statistically significant. To examine whether there was an associated depletion of tissue K+, we measured myocardial and skeletal muscle K+ content in control rabbits and in rabbits given aldosterone. The K+ contents are shown in Figure 2. There was no significant difference between control and aldosterone-treated rabbits. Because K+ depletion is known to cause a decrease in the abundance of Na+-K+ pumps in skeletal muscle,15 we also measured vanadate-facilitated [3H]ouabain binding. There were no significant changes in aldosterone-

Figure 1. Aldosterone dose and achieved plasma levels. Plasma levels of aldosterone (Ald) before infusion of aldosterone are indicated by solid bars, and plasma levels after a 7-day period of infusion in doses of 30, 50, and 100 μg/kg body weight per day are indicated by open bars. N indicates number of animals in each group. *Significant increase in plasma levels.

Figure 2. Aldosterone and tissue K+ content. The K+ content in the myocardium (myocard), soleus, and EDL muscles is shown. Open bars indicate K+ content in 6 control rabbits; solid bars, content in 6 rabbits infused with aldosterone for 7 days with 50 μg/kg body weight per day.
treated rabbits as compared with controls in the soleus muscle (202±14 versus 225±7 pmol/g wet weight) or in the extensor digitorum longum (EDL) muscle (140±8 versus 136±8 pmol/g wet weight). Similarly, treatment with aldosterone had no effect on K⁺-dependent pNPPase activity in soleus and EDL muscles (data not shown).

Effect of Aldosterone on \( I_p \)

We isolated myocytes from rabbits infused with ethanol vehicle and from the rabbits used in the series of experiments in which an appropriate dose of aldosterone was determined. We measured \( I_p \) in the myocytes using a Na⁺ concentration in the pipette solution ([Na]_{pip}) of 10 mmol/L. Figure 3 shows representative recordings of membrane currents during measurement of \( I_p \) in myocytes from a control rabbit and an aldosterone-treated rabbit. The mean \( I_p \) for myocytes from control rabbits and rabbits given 30, 50, or 100 μg/kg body weight per day is summarized in Figure 4. Mean \( I_p \) for myocytes from control rabbits was 0.91±0.09 pA/pF. There was no significant difference (P=0.06). These results do not definitively rule out an effect of aldosterone on the number of sarcolemmal Na⁺-K⁺ pump units. We used 2 additional methods to determine the effect of aldosterone on the abundance of pump units, vanadate-facilitated \(^3\text{H}\)ouabain and measurement of K⁻-dependent pNPPase activity. We measured vanadate facilitated \(^3\text{H}\)ouabain binding in intact myocardial samples isolated from 6 rabbits given aldosterone and from 6 control rabbits. The mean \( I_p \) of myocytes from rabbits treated with aldosterone was 1.61±0.06 pA/pF and mean \( I_p \) of myocytes from control rabbits was 1.77±0.04 pA/pF. There was no significant difference (P=0.06). These results do not definitively rule out an effect of aldosterone on the number of sarcolemmal Na⁺-K⁺ pump units.

Effect of Aldosterone on Na⁺-K⁺ Pump Concentration

The results shown in Figures 4 and 5 were obtained with a [Na]_{pip} similar to physiological levels of intracellular Na⁺. To examine whether aldosterone affects \( I_p \) when intracellular Na⁺ is at a level expected to nearly saturate binding sites, \( I_p \) was measured using a [Na]_{pip} of 80 mmol/L in 12 myocytes from 5 rabbits infused with aldosterone and in 16 myocytes from 5 control rabbits. The mean \( I_p \) of myocytes from rabbits treated with aldosterone was 21±0.36 pmol/g wet weight, whereas the mean concentration in control rabbits was 642±29 pmol/g wet weight. There was no significant difference. We measured K⁻-dependent pNPPase activity in crude homogenates of myocardium. The mean activity in myocardium isolated from 6 rabbits treated with aldosterone was 669±21 pmol/g wet weight, whereas the mean concentration in control rabbits was 642±29 pmol/g wet weight. There was no significant difference. We measured K⁻-dependent pNPPase activity in crude homogenates of myocardium. The mean activity in myocardium isolated from 6 rabbits treated with aldosterone was 0.97±0.05 μmol min⁻¹/g wet weight, whereas the mean activity in 6 control rabbits was 0.91±0.09 μmol min⁻¹/g wet weight. There was no significant difference between the groups.

Effect of Mineralocorticoid Receptor Blockade

To examine whether the classical mineralocorticoid receptor is involved in the effect of aldosterone on \( I_p \), we used the
mineralocorticoid receptor blocker spironolactone, comparing rabbits given aldosterone alone, those given spironolactone alone, and those given both aldosterone and spironolactone. Spironolactone was administered via osmotic minipumps in a dose of 200 μg/kg body weight per day. All rabbits were treated for 7 days. The mean serum K⁺ levels for the 3 groups are shown in Figure 6A. During the 7-day treatment period, serum K⁺ decreased by a similar amount in rabbits given combined aldosterone and spironolactone and rabbits given aldosterone alone. We conclude that spironolactone in the dose we used had no effect on serum K⁺.

We compared Iₚ of myocytes from rabbits given aldosterone alone, those given spironolactone alone, and those given both aldosterone and spironolactone in combination. Iₚ was measured using a Na⁺ pump of 10 mmol/L. Mean Iₚ values of myocytes from each of the 3 groups of rabbits are shown in Figure 6B. Mean Iₚ of myocytes from rabbits given spironolactone alone was similar to mean Iₚ shown in Figures 4 and 5 of myocytes from untreated control rabbits (0.34±0.02 versus 0.35±0.02 pA/μF). Spironolactone completely prevented the aldosterone-induced decrease in mean Iₚ. We conclude that the effect of hyperaldosteronemia on myocardi cardiac Na⁺-K⁺ pump function is mediated by the classical mineralocorticoid receptor.

**Effect of Aldosterone and Spironolactone on a*Iₚ**

Because treatment with aldosterone results in a decrease in Iₚ when [Na]ᵢₚ is near physiological intracellular levels, one would expect that treatment induces an increase in intracellular Na⁺ level. To examine this, we measured a*Iₚ in single intact papillary muscles isolated from 7 rabbits infused with aldosterone for 7 days and from 7 control rabbits. The mean a*Iₚ in papillary muscles from aldosterone-treated rabbits was 10.4±0.9 mmol/L, whereas the mean a*Iₚ in control papillary muscles was 7.0±0.4 mmol/L. Aldosterone induced a significant increase in a*Iₚ. We also measured a*Iₚ in papillary muscles from a third group of 4 rabbits treated with both aldosterone and spironolactone. Spironolactone prevented the aldosterone-induced increase in a*Iₚ (7.2±1.1 mmol/L). The effects of aldosterone and spironolactone on a*Iₚ are summarized in Figure 7A.

We have previously found that acute exposure of isolated papillary muscles to aldosterone in vitro enhances influx of Na⁺ via the Na⁺/K⁺/2Cl⁻ cotransporter. We next examined whether enhanced Na⁺ influx via the Na⁺/K⁺/2Cl⁻ cotransporter contributed to the increase in steady-state a*Iₚ observed in hyperaldosteronemic rabbits (Figure 7A). Aldosterone-induced Na⁺ influx can be detected as an increase in the rate of rise of a*Iₚ on Na⁺-K⁺ pump blockade with the fast-acting cardiac steroid DHO. We isolated single papillary muscles from 6 rabbits treated with aldosterone and from 5 control rabbits. We then recorded the rate of rise of a*Iₚ on superfusion with 500 μmol/L DHO. Figure 7B summarizes the time course of the recorded changes in a*Iₚ. There was no significant difference between the rate of rise in a*Iₚ in papillary muscles from rabbits treated with aldosterone and in papillary muscles from controls. This suggests that the aldosterone-induced increase in steady-state a*Iₚ is due to a reduction in extrusion of Na⁺ via the Na⁺-K⁺ pump rather than to an increase in Na⁺ influx.

**Discussion**

**Effect of Aldosterone on Na⁺-K⁺ Pump Function**

Two previous studies have examined the effect of aldosterone on activity of the Na⁺-K⁺ pump in the heart. Hegyvary reported that aldosterone administered to guinea pigs in vivo induces an increase in the activity of isolated myocardial Na⁺-K⁺ ATPase, and Ikeda et al reported that exposure of isolated intact cardiac myocytes to aldosterone in vitro
induces an increase in activity of the Na\(^+-\)K\(^+\) pump. These findings are difficult to reconcile with the aldosterone-induced decrease in sarcolemmal Na\(^+-\)K\(^+\) pump function demonstrated in the present study. It is important to consider details of the experimental evidence supporting such conflicting conclusions.

Hegyvary\(^{16}\) gave guinea pigs twice daily intraperitoneal injections of aldosterone for 2 weeks in a weight-adjusted dose \(\approx 3\)-fold higher than the daily dose in our study. The serum aldosterone levels achieved were not reported. However, given the intermittent mode of administration, it is likely that high peak levels were reached, possibly activating glucocorticoid as well as mineralocorticoid receptors. Because glucocorticoids induce an upregulation of the Na\(^+-\)K\(^+\) pump,\(^{15}\) such an increase in Na\(^+-\)K\(^+\) ATPase activity should not necessarily be taken to indicate a physiologically relevant effect mediated by the mineralocorticoid receptor.

Ikeda et al\(^{7}\) exposed cardiac myocytes to aldosterone for 72 hours and studied gene expression and function of the Na\(^+-\)K\(^+\) pump. Aldosterone was reported to cause an increase in Na\(^+\) influx. A decrease in myocyte Na\(^+\) content to levels below those of control myocytes after withdrawal of aldosterone was taken to indicate “unmasking” of functionally significant aldosterone-induced upregulation of the Na\(^+-\)K\(^+\) pump. The accuracy of this indirect approach is critically dependent on assumptions that are difficult to verify with certainty. In addition, it should be noted that, whereas physiologically relevant concentrations of aldosterone were used in some of the studies on gene expression, a concentration of 1 \(\mu\)mol/L was used in studies on function of the Na\(^+-\)K\(^+\) pump. At such concentrations, aldosterone is expected to have nonspecific effects.

We used the whole-cell patch clamp technique to control membrane voltage and the concentrations of intracellular and extracellular ligands during measurement of pump function in our study. Aldosterone induced a decrease in electrogenic Na\(^+-\)K\(^+\) pump current measured when [Na\(^+\)]\(_{in}\) was near physiological intracellular levels. The conclusion that aldosterone, as administered in our study, induces pump inhibition was strongly supported by the demonstration of an aldosterone-induced increase in free cytosolic Na\(^+\) activity in intact papillary muscles. A role of the mineralocorticoid receptor was indicated by the reversal of aldosterone-induced changes in pump current and \(a'_w\), by spironolactone.

**Effect of Aldosterone on Myocardial Na\(^+-\)K\(^+\) Pump Concentration**

Aldosterone had no effect on \(I_p\) measured using a [Na\(^+\)]\(_{in}\) expected to nearly saturate intracellular pump sites. Because \(I_p\) measured under similar conditions in oocytes accurately reflects the number of pump sites determined with the \[^{[3H]}\)ouabain binding technique,\(^{18}\) aldosterone appears to have no effect on the number of myocardial pump sites. Measurements of vanadate-facilitated \[^{[3H]}\)ouabain binding to intact myocardial samples in the present study support this conclusion. Because isoforms relatively insensitive to ouabain might not be detected by our measurements of \(I_p\) and \[^{[3H]}\)ouabain binding, we measured K\(^-\)-dependent pNPPase activity, an index independent of the sensitivity of Na\(^+-\)K\(^+\) pump isoforms to ouabain. Taken together, our findings indicate that aldosterone had no effect on the concentration of myocardial Na\(^+-\)K\(^+\) pump sites. The demonstrated decrease in pump function is consistent with an aldosterone-induced decrease in the apparent affinity of the pump for intracellular Na\(^+\).

**Metabolic Effects of Hyperaldosteronemia and the Na\(^+-\)K\(^+\) Pump**

Because hyperaldosteronemia can be associated with K\(^+\) depletion and because K\(^+\) depletion has been reported to affect the Na\(^+-\)K\(^+\) pump in skeletal\(^{15,17,19,20}\) and cardiac muscle,\(^{21}\) the possibility that K\(^-\) deficiency accounts for the decrease in \(I_p\) in our study should be considered. However, the decrease in serum K\(^+\) in our study was considerably less than the decrease usually associated with downregulation of the pump. Because skeletal muscles contain \(\approx 75\%\) of the total body K\(^+\) content,\(^{22}\) a major effect of aldosterone on K\(^+\) balance should also be reflected in the skeletal muscle K\(^+\) content. We did not find an aldosterone-induced reduction in K\(^+\) contents of skeletal muscle or myocardium. An effect of aldosterone on K\(^+\) balance is associated with a decrease in the abundance of Na\(^+-\)K\(^+\) pump units. There was no such decrease in skeletal or cardiac muscle in our study. It is also important to note that spironolactone completely abolished the effect of aldosterone on \(I_p\) without having any effect on the decrease in serum K\(^+\) that developed during treatment with aldosterone (see Figure 6). Finally, it should be noted that K\(^-\) depletion in rabbits increases rather than decreases electrogenic pump activity in cardiac myocytes.

Because hyperaldosteronemia can be associated with a decrease in levels of thyroid hormone\(^9\) and because hypothyroidism can reduce Na\(^+-\)K\(^+\) pump function in rabbit heart,\(^{24}\) the possibility that hypothyroidism accounts for the decrease in pump function in the present study should also be considered. However, given that thyroid hormone regulates synthesis of Na\(^+-\)K\(^+\) pumps,\(^{25}\) the absence of an effect of aldosterone on the abundance of Na\(^+-\)K\(^+\) pump units suggests that the aldosterone-induced decrease in \(I_p\) is not related to thyroid function. To obtain independent support for this, we measured levels of triiodothyronine and thyroxine in 6 rabbits before and after treatment with aldosterone. There was no detectable effect on thyroid function by aldosterone treatment (data not shown).

**Clinical Implications of Aldosterone-Induced Na\(^+-\)K\(^+\) Pump Inhibition**

Serum levels of aldosterone in patients with congestive heart failure are \(\approx 3\)-fold higher than levels in patients without heart failure. Both clinical\(^{26}\) and experimental\(^{27,28}\) evidence suggests that chronically elevated aldosterone levels have an adverse effect on the heart. High intracellular Na\(^+\) levels in the myocardium of patients with heart failure\(^{29}\) may at least in part be related to aldosterone-induced Na\(^+-\)K\(^+\) pump inhibition. Because of the steep, nonlinear dependence of intracellular Ca\(^+\) on the transmembrane Na\(^+\) concentration gradient,\(^{30}\) this is expected to cause a large increase in intracellular
Ca\textsuperscript{2+}. Cellular overload of Na\textsuperscript{+} and Ca\textsuperscript{2+} is believed to be important in the pathogenesis of cardiac arrhythmias,\textsuperscript{31} a common complication of congestive heart failure. Aldosterone-induced Na\textsuperscript{+}-K\textsuperscript{+} pump inhibition may also contribute to cardiac remodeling in heart failure, because pump inhibition can cause activation of key growth-related genes in cardiac myocytes\textsuperscript{32,33} and contribute to myocyte hypertrophy.\textsuperscript{33,34} The present study suggests that aldosterone receptor antagonists offer a rational therapeutic approach, a notion supported by recent reports of clinical benefits of such drugs.\textsuperscript{35,36}

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