Mechanisms for Aldosterone and Spironolactone-Induced Positive Inotropic Actions in the Rat Heart

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Abstract—Previously, we reported that aldosterone and spironolactone have inotropic effects in the isolated perfused heart. To address the mechanisms underlying these inotropic effects, we examined the effects of aldosterone and spironolactone on isolated cardiac myocyte shortening, intracellular calcium ([Ca^{2+}]), pH, and calcium-dependent actomyosin ATPase activity. Aldosterone significantly increased shortening in cardiac myocytes (8.0±1.0 versus 16.0±1.3%, \(P<0.01\)) but neither diastolic [Ca^{2+}] (61.0±1.1 versus 66.0±4.4 nmol/L) nor peak systolic [Ca^{2+}] (302±11 versus 304±17 nmol/L) was affected. Spironolactone-increased shortening was also not coupled with changes in peak systolic calcium; however, diastolic calcium was significantly increased by spironolactone. Aldosterone, but not spironolactone, increased pH from 7.23±0.03 to 7.59±0.02 (\(P<0.01\)); this was completely blocked by coadministration of 100 µmol/L of ethyl-isopropyl amiloride (EIPA), an inhibitor of the Na^+/H^+ exchanger (\(P<0.01\)). Consistent with this finding, aldosterone increased cytosolic sodium concentration ([Na^{+}]) from 9.2±0.15 to 11.4±0.2 mmol/L and produced a leftward shift in the pCa ATPase curve (pCa=5.82±0.02 versus 6.35±0.02, \(P<0.01\)) without affecting maximal myosin ATPase activity. Conversely, spironolactone, but not aldosterone, significantly increases maximal actomyosin ATPase activity (837±59 versus 355±52 nmol inorganic phosphate (P_i)·min^{-1}·g tissue^{-1}). Collectively, these data strongly suggest that the inotropic actions of aldosterone and spironolactone are caused by different mechanisms of action. Aldosterone appeared to increase inotropy primarily through increased cytosolic pH, whereas spironolactone increased myosin ATPase calcium sensitivity and diastolic calcium concentration. (Hypertension. 2004; 44:1-7.)

Key Words: mineralocorticoid ■ cardiac function ■ heart failure ■ calcium

The Randomized ALdactone Evaluation Study (RALES) demonstrated significant reductions in morbidity and mortality when spironolactone, the aldosterone mineralocorticoid receptor antagonist, was included in the treatment of heart failure. This study, along with many translational reports, strongly developed the concept that aldosterone may play an important, deleterious effect in human congestive heart failure. In addition, we have previously observed that aldosterone has rapid, inotropic effects in the isolated perfused rat heart; however, rapid, inotropic effects were observed as early as the 1960s. This rapid, inotropic effect of aldosterone almost certainly occurs through nongenomic mechanism(s).

Nongenomic actions of aldosterone were first identified by Wehling et al in smooth muscle cells. These actions were classified as nongenomic because of their speed, lack of inhibition by spironolactone, and independence from new protein synthesis. Based on these characteristics, nongenomic effects of aldosterone have been reported from a number of laboratories in renal epithelial cells, vascular smooth muscle cells, skeletal muscle cells, and colonic. The molecular basis of these nongenomic effects of aldosterone actions have been ascribed to changes in intracellular pH (pHi), intracellular calcium (Ca^{2+}), and intracellular sodium ([Na^{+}] in various tissues. Different effects on cytosolic pH and cytosolic calcium have been observed in different tissues. Although it has been observed that aldosterone does have rapid effects on protein kinase C activity in cultured cardiac cells, and that aldosterone has rapid effects on cardiac sodium–hydrogen exchange, the molecular mechanism of the rapid inotropic effect of aldosterone is still unclear. The spironolactone issue is less well defined and perhaps of greater interest because of the favorable results of the RALES trial. We previously observed that substantial inotropy could be observed with levels of spironolactone that were comparable to those achieved with this agent in standard clinical practice. Moreover, the inotropic effect of spironolactone was found to be additive to that of aldosterone when both agents were administered together. These studies strongly suggest that although aldosterone and spironolactone are similar in structure, they have independent mechanism(s) of actions. Therefore, the objective of the present study was...
to compare and contrast the responses of aldosterone and spironolactone at the cellular and organ levels to discern the molecular mechanisms underlying the inotropic effects of these agents previously reported.6

Methods

Animals
All procedures were approved by the Medical College of Ohio Institutional Animal Care and Use Committee and were conducted in accordance with the Guiding Principles in the Care and Use of Animals, as approved by the Council of the American Physiological Society. Male Wistar Kyoto rats were purchased from Harlan Sprague-Dawley (Indianapolis, Ind) at 10 to 11 weeks of age. All rats were provided food and water ad libitum and placed on a 12:12-hour light–dark cycle, with the light cycle occurring during the daytime.

Isolation and Culture of Adult Cardiac Left-Ventricular Myocytes
Adult, calcium-tolerant, left-ventricular cardiac myocytes were prepared from Wistar Kyoto rats (male; 12 weeks old) as previously reported.28–31 Cells were studied with a temperature-controlled chamber at 37°C and constant perfusion with either Krebs-Henseleit bicarbonate saline or Krebs-Henseleit HEPES saline aerated 95% O2 and constant perfusion with either Krebs-Henseleit saline except NaHCO3 was replaced with 20 mmol/L HEPES. The concentration of aldosterone used (10 nmol/L) was chosen because it was previously shown to elicit maximal contractility.6 For spironolactone at the cellular and organ levels to discern the molecular mechanisms underlying the inotropic effects of these agents previously reported.6

Intracellular Ca2+, Na+, and pH Measurements
Myocytes were loaded with Fluor-3-AM, sodium-binding benzofuran isophthalate, or 2,7’-bis(2-carboxylethyl)- or 5,6-carboxyfluorescein (BCECF) for the measurement of cytosolic calcium, cytosolic sodium, or pH. All of these fluorescent indicators were purchased from Molecular Probes (Eugene, Ore). The determinations of cytosolic calcium, pH, cytosolic Na+, and cellular contractility were performed as previously described.32–34

For some BCECF-loaded cells, different concentrations of ammonium chloride (NH4Cl) were used to determine the NH4Cl concentration needed to simulate aldosterone-induced intracellular alkalinization. The time course for intracellular alkalinization via NH4 Cl was rapid (reaching peak in 20 seconds after addition to the chamber) and persisted for ~1 to 2 minutes depending on the concentration. Based on in vitro testing on BCECF-loaded myocytes (data not shown), the concentration of 40 mmol/L NH4Cl was determined to achieve comparable alkalinization to that seen with aldosterone. Therefore, this dose was added to the atrial perfusate for subsequent working heart experiments.

Preparation of Cardiac Myofibrils for Ca2+-Activated Myosin ATPase Activity
Hearts removed from rats were retrogradely perfused with either vehicle or vehicle supplemented with either 10 nmol/L aldosterone or 1000 nmol/L spironolactone. Cardiac myofibrils were prepared according to the method described by Solaro et al.35 Assays were performed with the use of incubation conditions established by varying the total concentration of metals, salts, and ligands, maintaining ionic strength using the stability constants compiled by Fabiato,36 and were performed at pH 7.0 with 50 mmol/L imidazole, 50 mmol/L KCl, and 2 mmol/L MgATP. Myo-ATPase activity was determined by Fiske and Subbarow37 and subsequently modified by Tashima.39 All responses were normalized to pCa 9.0 (value set at 0.0) and pCa 4.0 (value set at 1.0).

Isolated Heart Studies
Isolated heart performance was measured using the Langendorff-Neely isolated working heart preparation as previously reported.40 After retrograde perfusion for 15 minutes at 80 mm Hg, anterograde perfusion was initiated at a preload of 15 mm Hg and an afterload of 70 mm Hg for the duration of each study.

Statistical Procedures
Initially, all data were tested for homogeneity of variance using a Levene test. If normally distributed, then ANOVA was performed. If data were nonparametric, then Kruskal-Wallis ANOVA was performed. Parametric homogeneous subsets were identified using the Scheffe post hoc test. For nonparametric data, subsets were identified using a Tamhane post hoc test for unequal variance. All statistical tests were performed using SPSS statistical software. The 5% level of confidence was arbitrarily used for assigning statistically significant differences.

Results

Isolated Myocyte Studies
The addition of aldosterone (10 nmol/L) significantly increased myocyte fractional shortening from (P<0.01; Figure 1a and 1b). This occurred without an increase in either diastolic or peak systolic [Ca2+]i, (Figure 1c and 1d). After the addition of 1000 nmol/L spironolactone, fractional shortening increased significantly (P<0.01; Figure 2a and 2b). As with aldosterone, this increase in fractional shortening occurred without an effect on peak systolic [Ca2+]i (Figure 2c and 2d). However, spironolactone significantly increased diastolic [Ca2+]i, and was, thus, associated with a smaller transient amplitude (Figure 2c and 2d).

Aldosterone had a rapid and dramatic effect on cytosolic pH. Specifically, addition of aldosterone increased pHi by 0.36 U relative to vehicle controls (Figure 3). The time course for aldosterone-induced alkalinization was rapid (reaching peak in 20 seconds) and persisted for ~1 to 2 minutes. The addition of spironolactone alone did not change pHi compared with vehicle. Moreover, spironolactone (1000 nmol/L) failed to block aldosterone-induced intracellular alkalinosis. Conversely, the addition of ethyl-isopropyl-amiloride (EIPA; 100 μmol/L), an inhibitor of the Na+/H+ exchanger, did prevent aldosterone-induced intracellular alkalinization (Figure 3). EIPA alone did not significantly alter pHi.

To examine whether aldosterone-induced intracellular alkalinosis was influenced by the [HCO3−], the aldosterone treatment experiments were also performed in cells bathed in buffer containing 20 mmol/L HEPES substituted for bicarbonate in the Krebs-Henseleit saline. Aldosterone induced alkalinosis was comparable when HEPES was substituted for bicarbonate (pH=7.55±0.01 versus 7.59±0.02). Moreover, in the presence of NaHCO3 and EIPA, aldosterone-induced alkalinosis was completely blocked, and pHi was comparable to that seen with EIPA alone (pHi=7.22±0.03 versus 7.21±0.06, P=NS). Consistent with aldosterone-induced alkalinosis and amiloride sensitivity, changes in intracellular [Na+], were also observed in the presence of aldosterone (Figure 4). On average, intracellular [Na+]i, was increased from a baseline level of 9.2±0.15 mmol/L to...
11.4±0.2 nmol/L after the addition of 10 nmol/L aldosterone (Figure 4, P<0.01).

**Isolated Heart Studies**

In the isolated heart system (Figure 5), perfusion with buffer supplemented with 10 nmol/L aldosterone elicited a 43% increase in stroke volume relative to vehicle control (176±2 versus 120±8 µL/g heart weight, P<0.01). Perfusion with buffer supplemented with 100 µmol/L amiloride (EIPA; N=6) blocked the aldosterone-induced positive inotropic effect (stroke volume 139±2 versus 176±2 µL/g heart weight, P<0.01), whereas EIPA alone (N=6) produced only a slight increase in stroke volume relative to vehicle (137±3 versus 120±8 µL/g heart weight, P<0.01).

As illustrated in Figure 3, 40 nmol/L ammonium chloride (NH₄Cl) produced an alkalinizing response comparable to 10 nmol/L aldosterone (7.6±0.04 versus 7.59±0.02, NS). To demonstrate the connection between intracellular alkalinization and inotropy, ammonium chloride (NH₄Cl) (which acutely alkalinizes the cytosolic pH by ≈0.2 pH units) was added to the atrial perfusate of isolated working hearts. As shown in Figure 5, NH₄Cl produced a similar increase in stroke volume as 10 nmol/L aldosterone (171±5 versus 176±2 µL/g heart weight). Interestingly, spironolactone (167±8 µL/g heart weight, N=6) and aldosterone treatment caused increases in stroke volume to a similar degree as that seen with an optimal dose of epinephrine (10 nM, 175±5 µL/g heart weight, N=6 experiments).

**Myofibrillar ATPase**

Relative to vehicle, myofibrils prepared from aldosterone-perfused hearts produced a leftward shift in the pCa–ATPase curves (pCa₅₀=5.8±0.1 versus 6.4±0.3, P<0.01; Figure 6a). However, compared with control, aldosterone was without effect on the Hill coefficient (2.2±0.2 versus 2.1±0.4). Conversely, myofibrils obtained from spironolactone-perfused hearts produced similar pCa₅₀ relative to vehicle controls (pCa₅₀=5.8±0.3 versus 5.8±0.1; Figure 6a). However, spironolactone significantly increased the Hill coefficient (Hill coefficient=2.2±0.2 versus 3.1±0.3, P<0.01). Interestingly, the pCa–ATPase curve from the aldosterone plus spironolactone perfusion produced both a leftward shift in the pCa₅₀ (6.4±0.1, P<0.01; Figure 6a) and a trend toward a steeper slope (Hill coefficient=2.7±0.3, P=0.06). Consistent with these observations were maximal ATPase activities for spironolactone-perfused myofibrils, which were 2.4-times higher compared with aldosterone (354±52 versus 836±65 nmol Pi·min⁻¹ per gram of tissue, P<0.01; Figure 6b).

**Discussion**

Recent studies have increased our collective interest in both the nongenomic effects of aldosterone and physiological effects of spironolactone. By contrasting the effects of aldosterone and spironolactone in isolated cardiac myocytes, we observed that aldosterone does not alter systolic or diastolic intracellular calcium, whereas spironolactone appears to actually decrease the magnitude of the calcium transient. This finding is consistent with tissue-specific changes in [Ca²⁺]i elicited by aldosterone.
sterone, but not spironolactone, produced a very substantial alkalinizing response. However, amiloride, the Na+/H+ exchange inhibitor, completely blocked the aldosterone-induced alkalosis. Consistent with these findings was an increase in [Na+]i after the administration of aldosterone (Figure 3).

EIPA was used to inhibit the Na+/H+ exchanger; however, at the high dose that we used in this report, it is possible that this agent had nonspecific effects. Interestingly, intracellular alkalinization from aldosterone was similar with either bicarbonate or HEPES-based buffers. This finding demonstrates that aldosterone-induced intracellular alkalinization did not require HCO₃⁻ in the media. Moreover, the intracellular sodium measurements also support that activation of the Na+/H+ exchanger is involved with aldosterone-induced inotropy. We would stress that our findings are quite consistent with other literature on this subject. Furthermore, as evidenced by Figure 4, aldosterone-induced inotropy can be mimicked with comparable degrees of cytosolic alkalinization achieved through ammonium chloride. In sum, the rapid inotropic effect of aldosterone appears to be entirely caused by its effects on pHᵢ, which elicits a leftward shift in the pCa myosin ATPase response.

The effect of spironolactone on inotropy may actually be of immediate clinical relevance. This agent caused a 2-fold increase in diastolic calcium without any change in systolic calcium concentrations. At this point, we comment on a potential shortfall of our use of the nonratiometric dye, Fluo-3, for the cytosolic calcium measurements. Because leakage can occur with fluorescence indicators, ratiometric dyes (eg, the BCECF and SBFI used for the pHᵢ and cytosolic Na experiments) are usually preferred. In particular, we have previously used Indo-1 to study calcium transients with our system. However, in these experiments in which we anticipated substantial increases in the pHᵢ in the aldosterone-treated cells, we chose to use Fluo-3 because it is relatively pH-insensitive while still giving extremely high signal-to-noise ratio. Despite our stable time controls, it is possible that
during the short-term experiments performed, some dye leakage occurred in the spironolactone group, and some increases in cytosolic calcium, especially systolic values, could have been missed. Although the significance of this increase in diastolic calcium is not yet known, this finding is similar to the 80 nmol/L increase in intracellular calcium reported in human bronchial epithelial cells elicited by spironolactone.21 Unlike aldosterone, spironolactone also did not alter the pCa50. However, in contrast, spironolactone produced a maximal ATPase response, which was 2.4-fold higher than that seen with aldosterone. Although it is appealing to attribute the changes in contractility to these increases in ATPase activity, at present we are left with only an interesting correlation. In simple terms, it appears that spironolactone causes changes in calcium, as well as in the molecular response to changes in calcium. Moreover, in contrast to aldosterone, it is clear that further work is necessary to fully understand the molecular mechanism by which spironolactone increases inotropy.

As we observed in our earlier report,6 the additive contractile effect of aldosterone with spironolactone suggests independent mechanisms of action for these agents. This hypothesis is further supported by the results on the current studies,
which demonstrated contrasting actions on diastolic intracellular calcium, pH, calcium-activated myosin ATPase activation, and maximal myosin ATPase activity. At present, we do not know the molecular basis for the effects of spironolactone on cytosolic calcium and myofibrillar calcium sensitivity. One possibility is that these effects may be caused, at least in part, by effects of its primary metabolite, canrenonic acid.\(^{54}\) It may be that either spironolactone, which does achieve extremely high concentrations, and/or canrenoic acid signals through the sodium pump in an analogous fashion to ouabain at extremely high concentrations, and/or canrenoic acid signals through the sodium pump in an analogous fashion to ouabain.

Tant implications for the clinical treatment of heart failure (the Randomized Aldactone Evaluation Study [RALES]).\(^{33,53}\) However, this possibility was not addressed in the current report. Clearly, further investigation of these molecular mechanisms that may have important implications for the clinical treatment of heart failure must be pursued.

Acknowledgments

The authors dedicate this work to the memory of our esteemed colleague Roberto Franco-Saenz, MD, a dedicated physician, brilliant scientist, and loyal friend. Descanse en la paz mi amigo.

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Hypertension. published online October 4, 2004;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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