Direct Aldosterone Action on Mouse Cardiomyocytes Detected with Atomic Force Microscopy

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Key Words

Spironolactone • Cardiac hypertrophy • Cellular volume • Plasma membrane surface • Membrane proteins • Nanotechniques

Abstract

There is growing evidence that aldosterone acts on heart where it causes cellular remodeling and hypertrophy. Since it is still unclear whether aldosterone directly acts on cardiomyocytes or indirectly, by an altered electrolyte balance in the organism, we applied atomic force microscopy (AFM) in primary cultures of neonatal mouse cardiomyocytes to measure hormone-induced changes in cell volume and plasma membrane surface. AFM measures cell volume and, at the same time, provides quantitative information on cell surface properties. Neonatal mouse cardiomyocytes were cultured for 28 hours in absence or presence of 100 nM aldosterone. Spironolactone was applied as a selective aldosterone receptor antagonist. At the microscopic level, single cell volume and single cell surface were found unchanged by aldosterone. However, nanoscopy of the cell surface, i.e. analysis of the plasma membrane at the

nanometer level, revealed a specific increase in plasma membrane nano-enfoldings (roughness). This aldosterone-mediated increase in cell surface roughness was completely prevented by spironolactone. We conclude: (i) Aldosterone directly acts upon cardiomyocytes. (ii) At the microscopic level, no changes of cell volume and cell surface are detectable. (iii) At the nanoscopic level, aldosterone increases plasma membrane roughness. These nanometer changes, detectable only with AFM in cells scanned in fluid after fixation under physiological conditions, indicate plasma membrane remodeling of cardiomyocytes by mineralocorticoids.

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Introduction

Aldosterone, the major mineralocorticoid hormone synthesized in the adrenal cortex, plays an essential role in water and electrolyte homeostasis. By acting on mineralocorticoid receptors in kidney, aldosterone causes sodium retention and a subsequent increase in blood volume.

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Aldosterone excess causes hypertension which is known to have adverse effects on the cardiovascular system. However, there is increasing evidence that aldosterone has possibly direct effects on the cardiovascular system that are independent of blood pressure. Endothelial cells directly respond to mineralocorticoids by swelling [1] and stiffening [2]. Also the heart apparently responds to aldosterone. Patients with primary aldosteronism have an increased risk of left ventricular hypertrophy compared to patients with essential hypertension of comparable severity [3]. The Randomized Aldactone Evaluation Study (RALES) revealed beneficial effects of spironolactone, a receptor-antagonist of aldosterone, on the morbidity and mortality in patients with heart failure at concentrations that did not show effects on the blood pressure [4]. Continuous aldosterone infusion in rats on a high salt diet causes accumulation of both perivascular and interstitial collagen, again prevented by a low dose of spironolactone [5]. Additionally, aldosterone was shown to be involved in structural remodeling of arteries [6] and in endothelial dysfunction [7] regardless of its effect on blood pressure. Accordingly, the mineralocorticoid receptor (MR) was shown to be expressed in endothelial cells, in vascular smooth muscle cells and in cardiomyocytes [8]. Furthermore, a local aldosterone production was found in tissues other than the adrenal cortex, such as vessels [9] and the heart [10].

However, it has been yet unclear to what extent aldosterone directly acts upon cardiomyocytes. Aldosterone increases the biosynthesis and/or modulates the activity of several membrane transporters and other proteins of cardiomyocytes [11, 12]. It is known to increase the number of angiotensin II (AT1) receptors in cardiac cells [13] and to stimulate the angiotensin-converting-enzyme (ACE) in cardiomyocytes [14]. This, however, raises the question of how much of the aldosterone-induced fibrosis and hypertrophy is mediated by an enhancement of the effects of Ang II. Furthermore, aldosterone inhibits inducible nitric oxide synthase (iNOS), which may contribute to virtually all of the aldosterone induced cardiac effects [15]. Additionally, it has been shown that aldosterone increases Ca^{2+} currents across cardiomyocyte membranes [16], thereby increasing the cytosolic concentration of a crucial transmitter in hypertrophic signaling [17]. However, the results of studies that apply cell culture in determining aldosterone-induced hypertrophy, are still controversial [18, 19].

Usually, cell function can be associated with structural changes in the cardiac myocyte phenotype. In the present study we aimed to detect such phenomena

Fig. 1. RT-PCR analysis of mineralocorticoid receptor mRNA in murine cardiomyocytes. Lane M: 100 bp marker; Lane 1: 563 bp fragment of MR amplified from cardiomyocytes RNA.

caused directly by aldosterone. For this purpose we analyzed volume and surface of cultured neonatal cardiomyocytes in response to aldosterone applying atomic force microscopy (AFM). AFM is a nanotechnology that allows the analysis of structural plasma membrane changes at the nanometer level. In a first step we measured, at the microscopic level, single cell volume and single cell surface of neonatal mice cardiomyocytes. In a second step we measured, at the nanoscopic level, putative changes in plasma membrane surface mediated by aldosterone exposure. We analyzed small cell membrane areas in terms of surface nano-enfoldings (roughness). The height resolution of the AFM is less than 1 nm [20]. As proteins protrude from the surface of the plasma membrane by up to 20 nm [21], any changes in the plasma membrane protein pattern could be reflected by such measurements.

Materials and Methods

Cardiomyocyte cell culture

Cardiomyocytes were isolated from neonatal mice at post-natal day 1. All investigations conform with the declaration of Helsinki and were approved by the local animal care committee. Mice were killed by cervical dislocation. Hearts were obtained and washed in DMEM (supplemented with glucose (1g/l) and L-glutamine, Gibco, Karlsruhe, Germany, buffered with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma-Aldrich, Steinheim, Germany)). The hearts were cut into pieces of about 1 mm³ in size and were then digested by enzymatic treatment using (i) collagenase type II at a final con-

Fig. 2. Single cardiomyocyte volume. Cells were exposed to either the solvent (Control), to aldosterone (Aldo) or to aldosterone plus spironolactone (Aldo + Spiro) for the time of cultivation (28 h). No differences in single cell volume are evident between the differently treated groups. The numbers in columns indicate the number of analyzed images.

centration of 0.219 mg/ml (Worthington, St. Katharinen, Germany) and (ii) pancreatin at a final concentration of 0.857 mg/ml (Gibco) at 37°C for 10 min. Enzymes were dissolved in DME medium at pH 7.4 (DMEM powder purchased from Sigma-Aldrich and supplemented with 0.13% NaHCO_3). The resulting cell suspension was transferred into a tube filled with newborn calf serum (Gibco) and spun down. These steps were repeated 5 times. Cells were then centrifuged and preplated for three hours in order to allow attachment of non-myocytes such as fibroblasts. The cardiomyocytes which remained in suspension were then plated on fibronectin (human plasma fibronectin, 25 μg in 1 ml phosphate buffered saline (PBS), Boehringer, Ingelheim, Germany) coated plastic coverslips placed in 12-well multi-dishes filled with plating medium (67% DMEM supplemented with glucose (1g/l) and L-glutamine, Gibco; 17% M199 supplemented with L-glutamine, Gibco; 10% heat inactivated horse serum, Gibco; 5% heat inactivated fetal calf serum, Gibco; 50 IU/ml penicillin; 50 IU/ml streptomycin). 17 h after plating cultured cardiomyocytes were washed three times with PBS and the plating medium was replaced by serum-free maintenance medium (78% DMEM and 20% M199, each supplemented as mentioned above; 50 IU/ml penicillin; 50 IU/ml streptomycin). Chemicals were added to plating and maintenance medium as appropriate. Aldosterone (d-aldosterone, Sigma-Aldrich) was dissolved in ethanol (stock solution = 1 mmol/l, stored at 4°C for two weeks) and used at a final concentration of 100 nmol/l. Spironolactone (ICN Biochemicals GmbH, Eschwege, Germany) was dissolved in ethanol (stock solution = 1 mmol/l) and applied at a final concentration of 1 $\mu\text{mol/l}$. Spironolactone was added 10 minutes prior to aldosterone to make sure that virtually all mineralocorticoid receptors were occupied before cells were exposed to the agonist (aldosterone). In corresponding control experiments the solvent alone (ethanol 0.1%) was added to the media. 28 h after plating, cardiomyocytes were fixed with glutaraldehyde (final concentration = 0.5%) gently added to the medium. This procedure was performed in the incubator to minimize irritations by

changes in temperature or CO_2 concentration. Then, the medium was replaced with HEPES-buffered solution (mmol/l: 140 NaCl; 5 KCl; 1 MgCl_2 ; 1 CaCl_2 ; 10 HEPES; pH = 7.4) and cardiomyocytes were stored in this HEPES buffer at 4°C.

AFM measurements

AFM [22] was performed in fluid in order to maintain structure and volume of cardiomyocytes. The plastic coverslips carrying the cells were attached to object slides with instant glue. Care was taken to keep the cells in fluid at all times. AFM was performed in contact mode using a Nanoscope III BioScope-AFM (Digital Instruments, Santa Barbara, California, USA). V-shaped silicon nitride cantilevers with spring constants of 0.06 N/m (Digital Instruments) were used mounted in the commercially available fluid cell (Digital Instruments). Images (512 $\times$ 512 pixels) were obtained with scan sizes of 10,000 μm^2 at a scan rate of 5 Hz. This means that each pixel covers an area of 200x200 nm. Further settings were: height mode, gains between 3 and 7; interaction force between AFM tip and sample surface less than 5 nN. 15 to 20 images were obtained from individual samples and analyzed using the Nanoscope III software (Digital Instruments, software No. 5.12r3). Each image, neither filtered nor smoothed, was plane-fitted (order 1) and, if necessary, zoomed in to exclude non-confluent areas.

AFM cell volume and surface analysis

The method of cell volume determination by AFM has been described in detail [23]. The data required for volume calculation is included in the three-dimensional images. The volume of the total image was analyzed using appropriate software (Digital Instruments). Mean single cell volume was obtained by dividing the total volume per image by the number of cells in the image.

Scanning a sample provides surface profiles. In order to scan a sample the desired size of the image area has to be defined, e.g. 100 μm $\times$ 100 μm = 10,000 μm^2 . When the coverslip is covered with myocytes the total surface of the image in-

Fig. 3. Single cell z surface. Cells were exposed to either the solvent (Control), to aldosterone (Aldo) or to aldosterone plus spironolactone (Aldo + Spiro) for the time of cultivation (28 h). Results of unpaired Student's t-test are given. The numbers in columns indicate the number of analyzed images. The insert gives a cell model consisting of cubes to illustrate the part of the surface the single z surface represents. As the difference between the single cell area (equivalent to the dark grey areas) and the single cell surface (sum of dark and light grey areas), the single cell z surface represents the light grey areas, that means the surface orientated in the z-axis.

creases (since the 'image area' is two-dimensional while the cell surface area is three-dimensional). Single cell surface was obtained by dividing the total surface of the image by the number of cells per image. Single cell area was calculated by dividing the image area by the number of cells per image. Then, we analyzed single cell z surface. This value represents the z-membrane (area) due to the z-dimension (height) of cells. This is explained in the insert of figure 3 that shows a model consisting of cubes. Subtraction of single cell area (equivalent to the dark-grey areas) from single cell surface (sum of dark-grey and light-grey areas), results in single cell z surface (light-grey areas). Since AFM height resolution is in the sub-nanometer range [20, 24] even minute alterations in the z-axis (height) can be detected.

The membranes of both the cell center and the cell periphery were investigated separately by zooming in cell areas of about 4 µm² in size. Only plane areas were chosen in order to determine the following parameter. The total surface of the analyzed cell area was determined and normalized to an analyzed cell area of 1 µm² in size (surface per 1 µm² cell area).

RT-PCR analysis of mineralocorticoid receptor mRNA

Total RNA was prepared from *Mus musculus* cardiomyocytes by mechanical homogenization, phenol-chloroform-isoamyl alcohol extraction, ethanol precipitation and an additional LiCl precipitation step as previously described [25]. RNA was quantified and its quality assessed by spectrophotometry. To purify RNA from contaminating DNA, it was incubated with 20 U DNase I (MBI Fermentas, St. Leon-Rot, Germany) per microgram RNA for 30 min at 37°C. Five micrograms of DNase I digested and phenol-chloroform-isoamylalcohol extracted total RNA was used for each reverse

transcription reaction. Complementary DNA (cDNA) synthesis was performed with Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions and using 500 ng oligo(dT)15 (MWG Biotech, Ebersberg, Germany) per reaction (20 µl) as first strand primer. A 563 bp fragment of *M. musculus* mineralocorticoid receptor was amplified by polymerase chain reaction (PCR) using the primers 5-AGCTCTTCTTCATGACAGATGATAGG-3 and 5-GGCTACCACAGTCTCCCTGAAGG-3. The reaction mixture consisted of 3 µl cDNA, 1.25 U Taq DNA polymerase (Eppendorf, Hamburg, Germany), 200 nmol/l of each primer, 200 µmol/l dNTPs, 50 mmol/l KCl, 10 mmol/l Tris-HCl pH 8.3, 1.5 mmol/l MgCl₂ in a final volume of 50 µl. PCR was performed in a thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) with an initial denaturation at 94°C for 5 min, 35 cycles of denaturation (94°C, 1 min), annealing (56°C, 1 min), and synthesis (72°C, 1 min and 30 s) and 15 min of final extension at 72°C. Reverse transcription reactions with water added instead of reverse transcriptase were used as negative control templates. PCR products were resolved on a 1.5% agarose gel in 0.5x TBE (44.5 mmol/l Tris, 44.5 mmol/l boric acid, 1 mmol/l EDTA), stained in 1x SYBR Gold (Molecular Probes, Leiden, Netherlands) in 0.5x TBE and photographed using an UV transilluminator.

Statistics

Mean data of experiments are given ± standard error of the mean (SEM). Statistical significance was tested applying the unpaired Student's t-test. Data were considered to be statistically significant when $P < 0.05$. The number of the analyzed images is given in the inserts of the figure columns.

Fig. 4. AFM images and cell profiles of cardiomyocytes. **A:** Cardiomyocytes cultured for 28 h under control conditions. **B:** Cardiomyocytes cultured for 28 h with aldosterone. On the left: Top view of cardiomyocytes. Lines correspond to the respective profiles, triangles mark cell border. Squares correspond to the respective magnification. On the right: cell profiles and magnification of the cell center. The surface per 1 μm^2 cell area and the surface roughness are increased under aldosterone treatment.

Results

In a first step we tested whether neonatal mouse cardiomyocytes express the mineralocorticoid receptor (MR). As shown in figure 1, RT-PCR reveals that the MR mRNA is expressed in the preparation we used.

In AFM experiments we analyzed volume and surface of neonatal cardiomyocytes under the following three conditions: (i) Cells were cultured for 28 h under control conditions, (ii) cells were cultured for 28 h in the presence of 100 nmol/l aldosterone and (iii) cells were cultured for 28 h, treated with both aldosterone and its competitive receptor antagonist spironolactone.

In a first approach, we intended to test for aldosterone responses on the single cell level. We, therefore, examined cell volume and cell surface. As shown in figure 2, single cell volume of cardiomyocytes did not differ significantly between control and hormone-exposed cells. Single cell volume of control cells averaged 2048 ± 43.4

fl ($n=48$). In aldosterone treated cells, single cell volume was shown to be 2053 ± 46.1 fl ($n=65$) and with aldosterone plus spironolactone 2078 ± 76.7 fl ($n=37$). We also did not detect changes in the single cell surface caused by aldosterone. Three-dimensional cell surface analysis of control cells was $1130 \pm 29.7 \mu\text{m}^2$. Upon aldosterone treatment, cell surface was $1113 \pm 30.1 \mu\text{m}^2$ and in cells, treated with aldosterone plus spironolactone, surface was $1170 \pm 42.3 \mu\text{m}^2$ (same number of analyzed images as for volume analyses). Two-dimensional analyses of the single cell area also did not show any significant differences. Cell area of control cells was $1058 \pm 28.8 \mu\text{m}^2$, that of aldosterone treated cells was $1029 \pm 28.5 \mu\text{m}^2$ and that of cells treated with aldosterone plus spironolactone was $1099 \pm 41.9 \mu\text{m}^2$ (number of analyzed images as above).

Thus, at first sight, aldosterone does not change surface and volume of cardiomyocytes. However, a look at the three-dimensional data of the cell surface at nanoscale

Fig. 5. Detailed membrane surface (surface per 1 μm^2 cell area). A: Analyses of the cell centers. B: Analyses of the cell peripheries. Cells were exposed to either the solvent (Control), to aldosterone (Aldo) or to aldosterone plus spironolactone (Aldo + Spiro) for the time of cultivation (28 h). Results of unpaired Student's t-test are given. The numbers in columns indicate the number of analyzed cut-outs.

disclosed some significant changes. The reason is as follows: Two-dimensional cell surface analysis includes the large scatter among different cells. Since cells are rather flat, any changes in the z-axis (third dimension) are 'hidden' in this scatter. This led us to analyze the 'z-membrane' which represents the difference in surface between the two- and the three-dimensional cell surface. Results are shown in figure 3. Single cell z surface (z-membrane) averaged $72 \pm 1.5 \mu\text{m}^2$ under control conditions. After aldosterone treatment z-membrane averaged $84 \pm 2.3 \mu\text{m}^2$. This significant increase was inhibited

by spironolactone ($70 \pm 2.2 \mu\text{m}^2$). Thus, we concluded that aldosterone does indeed induce a significant increase in surface of cardiomyocytes. This increase, however, occurs at the nanometer level of the plasma membrane and is thus not detectable with overall cell surface measurements. Figure 4 illustrates these arguments. Surface profiles indicate that plasma membrane is more 'rough' after aldosterone treatment. The insets in figure 4 show 'zoom in profiles' that support this view. To test whether such structural membrane changes occur randomly distributed across the cell surface, we

analyzed two different cell regions, the cell center and the cell periphery. At the cell center (figure 5A) the surface of $1 \mu\text{m}^2$ of analyzed cell area was $1.0198 \pm 0.00107 \mu\text{m}^2$ under control conditions. Aldosterone increased the surface to $1.0310 \pm 0.00151 \mu\text{m}^2$. This significant effect was inhibited by spironolactone ($1.0188 \pm 0.00114 \mu\text{m}^2$). The analysis of the cell periphery (figure 5B) revealed similar findings (control: $1.0173 \pm 0.00090 \mu\text{m}^2$, aldosterone: $1.0311 \pm 0.00133 \mu\text{m}^2$, aldosterone and spironolactone: $1.0157 \pm 0.00068 \mu\text{m}^2$).

Taken together, plasma membrane surface increases significantly in response to aldosterone, detectable only by using a nanoscopic approach.

Discussion

Cell volume measurements applying AFM are well established. This method offers the chance to measure the volume of adherent cells independent of cell shape [23]. Experiments scanning cells fixed in culture medium and maintained in buffer during measurements lead to images with high reliability [26]. Nevertheless, detailed cell surface analyses have been difficult. Simpson established a method to determine the surface area, called "single cell area" in the present study, by tracing digitized cell images with a LED cursor and by applying a special image analysis software [27]. This method combined with morphometric measurements and evaluation of expression of hypertrophy marker genes has been used to draw conclusions regarding cardiac cell growth. However, surface area analysis does not include the third dimension as it is just a two-dimensional projection of the cell on the ground. Measurements of cell capacitance as a measure for plasma membrane surface are usually applied in electrophysiological experiments [16, 28]. This procedure, however, cannot provide information about plasma membrane structure whereas AFM, as a scanning probe microscope, can provide surface profiles at three dimensions and thus makes analyses of adherent cells possible. However, it needs to be taken into account that only the surface, facing the scanning AFM tip, can be measured. AFM allows various approaches to analyze a surface. On the one hand, the three-dimensional cell surface can be analyzed microscopically (i.e. at μm scale), on the single cell level. On the other hand, the surface can be analyzed nanoscopically (i.e. at nanometer scale), on the plasma membrane level. This can disclose membrane changes at the low nanometer level, usually not detectable with any other technique. Another comparable

method is scanning electron microscopy. Although it offers an excellent lateral resolution, resolution in the vertical z-axis is limited by sample surface coating and is, thus, inferior to AFM which does not need any surface treatment.

Over the past years, direct effects of aldosterone on heart structure and function gained attention due to the fact that aldosterone apparently participates in pathophysiological processes of the cardiovascular system. Attention was drawn to the finding that the mineralocorticoid receptor antagonist spironolactone, in concentrations that do not markedly affect blood pressure, has beneficial influence on mortality and morbidity of patients suffering from heart failure [4]. These and other findings [3, 5, 29-31] indicate an effect of aldosterone on the heart that is independent from its effect on blood pressure. However, direct effects of aldosterone on cardiac myocytes have been barely shown.

In the present study we detected a direct effect of aldosterone on cardiomyocytes. This effect was blocked by spironolactone, indicating that classical MR is involved in the hormone's action. MR was shown to be expressed in cardiomyocytes [8, 32], which we were able to confirm for the cells we used in the present study. We analyzed volume and surface of cultured neonatal cardiomyocytes in response to a 28 h incubation with aldosterone. Cell volume did not change in response to the hormone and also, at first sight, we found no change in cell surface if a whole cell was analyzed. However, a closer look, using the nanoscopic capabilities of the AFM, revealed a subtle cardiomyocyte surface increase caused by apparent membrane enfoldings at nanometer scale. Taken together, there is no change in surface detectable at the whole cell level due to the scatter between cells. However, analysis of plasma membrane surface of individual cells at the nanoscopic level clearly shows an increase in surface roughness elicited by aldosterone treatment.

How to explain this increase in surface roughness?

It could be explained by an augmentation of the plasma membrane which subsequently leads to enhanced nano-enfoldings. The augmentation in cardiomyocyte membrane could be due to an aldosterone-induced incorporation of membrane containing proteins such as ion channels, transporters, pumps and receptors. Indeed, aldosterone is known to increase the number of cardiac AT 1 receptors in aldosterone-salt treated rats [13]. In cultured cardiac myocytes it stimulates Na^+/K^+ ATPase α -1 subunit mRNA synthesis and protein accumulation [33] in a rapid as well as in a sustained manner. The expression levels of the Na^+/H^+ exchanger isoform 1

(NHE-1) [34] as well as those of L-type and T-type calcium channels [16, 35] are increased after 24 h of aldosterone treatment.

Furthermore, an increase in apparent membrane nano-enfoldings could also reflect the accumulation of aldosterone-induced proteins themselves since AFM height resolution for biological membranes is in the range of 1 nm and plasma membrane proteins protrude up to 20 nm from the membrane [21]. However, images taken from whole cells do not allow to distinguish between nano-enfoldings of the plasma membrane lipid bilayer or membrane protein protrusions. Such a distinction is feasible only after plasma membrane isolation [36]. In this study, we put the focus on whole cells instead of membrane patches. Therefore, the question remains open whether the change in membrane structure, induced by aldosterone, is caused by an augmentation of the membrane, by the accumulation of membrane proteins themselves or by both of them.

Finally, aldosterone-mediated rearrangements of the cytoskeleton could also influence the membrane surface of the cardiomyocytes. However, such effects are currently unknown.

Our findings may also help to answer the question whether aldosterone directly induces cardiac hypertrophy. Patients with primary aldosteronism have an increased risk of left ventricular hypertrophy compared to patients with essential hypertension of comparable severity [3]. Serum aldosterone concentration is correlated to left ventricular mass [37]. Conversely, left ventricular hypertrophy could not be verified to be prevented by spironolactone, at a concentration that leaves blood pressure unaffected [5]. It was only prevented at spironolactone concentrations that decreased blood pressure. This finding again supports an indirect hypertrophic mechanism via haemodynamic effects. Some of the aldosterone-induced mechanisms are known to be involved in cardiac hypertrophy. Aldosterone augments endothelin-1-induced cardiac myocyte hypertrophy [19]. Calcium is known to be a crucial transmitter in hypertrophic signaling [17] and Ang II is well known to have growth stimulatory effects

[38]. NHE-1 is also known to be involved in hypertrophy [34]. However, studies in cultured myocytes exposed to aldosterone revealed controversial results. Oshima et al. treated neonatal rat cardiac myocytes with 100 nM aldosterone for 24 h. Measuring cell surface and upregulation of hypertrophic proteins did not support the view that hypertrophy was directly caused by aldosterone [19]. Also, Sato and Funder [16, 39] as well as Benitha and Vassort [16, 39] could not detect a direct hypertrophic effect of aldosterone. In contrast, Karmazyn et al. successfully demonstrated hypertrophy after 24 h treatment with 100 nM aldosterone [34] which was confirmed by Okoshi et al. [18].

Other studies measured cell volume as a determinant of myocyte hypertrophy [40]. In our experiments cell volume did not change in response to aldosterone. This argues against a hypertrophic effect of aldosterone under the applied conditions. Perrier et al. demonstrated that electrical remodeling precedes cellular hypertrophy [41]. They showed that, during the hypertrophic process, aldosterone first induces an increase in the total number of Ca^{2+} channels before hypertrophy occurs. That means that the number (or activity) of channels per defined surface area is increased. Later in the hypertrophic process, when the cell is enlarged, the number of channels per defined surface area goes back to normal (while the total number of channels is still increased). These observations are in agreement with the present study demonstrating changes in plasma membrane nanostructure.

In conclusion, aldosterone directly affects the nanostructure of the cardiomyocyte. This direct effect is mediated by classical intracellular mineralocorticoid receptors. Blockade by selective antagonists prevents the direct aldosterone action on the cardiomyocyte.

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