

Aldosterone receptor sites on plasma membrane of human vascular endothelium detected by a mechanical nanosensor

L. Wildling · P. Hinterdorfer · K. Kusche-Vihrog ·
Y. Treffner · H. Oberleithner

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Abstract The mineralocorticoid hormone aldosterone acts on target cells of kidney, colon, and the cardiovascular system through genomic and nongenomic pathways. Although the classical intracellular mineralocorticoid receptor plays a key role in mediating both pathways, it is unclear whether there are specific aldosterone receptors located on the cell surface. To search for such sites in vascular endothelium, we used an atomic force microscope (AFM) which measures unbinding forces based on single molecular recognition between an aldosterone-loaded AFM tip and the cell membrane. Aldosterone was tethered covalently via linker molecules to an AFM tip. Human endothelial cells (EA.hy926) were grown in culture and studied in buffer at 37°C. Using the aldosterone-functionalized AFM tip as a mechanical nanoscale indenter, unbinding forces could be measured at randomly chosen sites of the plasma membrane. Sites with strong interactions between AFM tip and cell surface could be identified exhibiting unbinding forces of about 65 pN. The binding probability between the aldosterone-loaded tip and the cell surface at selected membrane sites was $53 \pm 7.2\%$. Addition of an excess supply of aldosterone to the bath solution blocked the binding of the aldosterone-loaded tip to the cell surface.

The binding probability was reduced to $8.0 \pm 1.8\%$ when an excess supply of aldosterone was added to the bath. However, it was not influenced by the addition of spironolactone or dexamethasone. We conclude that aldosterone receptor sites exist on the cell surface of vascular endothelial cells distinct from the classical mineralocorticoid receptors and insensitive to glucocorticoids. Binding of aldosterone to these receptors initiates an intracellular signaling cascade that precedes the classical genomic response and most likely participates in the control of vascular resistance.

Keywords Spironolactone · Nongenomic · Dexamethasone · Mineralocorticoid receptor · Atomic force microscopy

Introduction

The mineralocorticoid aldosterone belongs to the class of steroid hormones which, after binding to intracellular receptors, trigger an intracellular signaling cascade including transcription of hormone-specific genes which results in de novo synthesis of proteins and respective changes in the function of the target cells. Besides this genomic action of aldosterone, it has been realized in the late 1980s that the hormone can in parallel elicit rapid changes in cellular function incompatible with a slow genomic action [16, 25, 36]. This nongenomic hormone response characterized by its fast onset and its insensitivity to blockers of gene transcription has been found increasingly in many different tissues, usually preceding the classic genomic response [3, 21]. Early observations in kidney tubules suggested that classic intracellular mineralocorticoid receptors play also a role in mediating the rapid nongenomic response [25]. Such

L. Wildling · P. Hinterdorfer
Institute of Biophysics, University of Linz,
Linz, Austria

K. Kusche-Vihrog · Y. Treffner · H. Oberleithner
Institute of Physiology II, University of Münster,
Münster, Germany

H. Oberleithner (✉)
Institute of Physiology II,
Robert-Koch-Str. 27b,
48149 Münster, Germany
e-mail: oberlei@uni-muenster.de

rapid nongenomic effects, mediated by the classic cytosolic receptors, were shown later on in a number of other tissues (for reviews, see [8, 9]). In the 1990s, mainly Wehling's group searched for specific aldosterone receptors in the plasma membrane of target cells. Although in a number of elegant papers they found some evidence that aldosterone was bound to plasma membranes [33–35], the case for a specific membrane receptor remained circumstantial.

The current concept of rapid nongenomic actions of aldosterone is mainly based on the presence of classic mineralocorticoid receptors which can serve both as transcription factors and, in parallel, as cytosolic proteins which participate in intracellular signaling cascades in a manner similar to those described for the cellular actions of estrogens [27].

Nevertheless, the participation of classic mineralocorticoid receptors in both genomic and nongenomic actions of aldosterone does not exclude the possibility that aldosterone could interact with target molecules directly at the cell surface. For example, following exposure to aldosterone, endothelial cells respond within 1 min with significant swelling which cannot be blocked by the mineralocorticoid antagonist spironolactone [22, 24]. This observation suggests that, apparently, an aldosterone-triggered mechanism exists independent of the classic intracellular receptors.

Atomic force microscopy can be successfully applied for the detection of plasma membrane microdomains provided that model membranes and defined membrane proteins are used [12]. This is not the case in the present study where living cells with still unknown receptor sites were investigated. Therefore, in search of putative aldosterone-specific plasma membrane receptor sites, we performed unbinding force measurements [17] on vascular endothelial cells. Aldosterone was covalently tethered to the nanoscale tip of an atomic force microscope and brought into physical contact with the plasma membrane of living endothelial cells, thereby enabling binding of the aldosterone molecules on the atomic force microscope (AFM) tip to the cell surface. Upon retraction of the tip, unbinding forces between the tip and the cell could be quantified. The results show the presence of aldosterone interaction sites on the cell surface and their insensitivity to spironolactone. It is possible therefore that these interaction sites are the structural basis for the first functional response of a target cell to aldosterone.

Materials and methods

Endothelial cell culture

Human endothelial cells (EA.hy926; [6]) were grown in culture as previously described [13, 19]. In brief, cells

(passage p32) were cultivated in T₂₅ culture flasks using Dulbecco's modified essential medium (DMEM; Invitrogen, Karlsruhe, Germany) with addition of NaHCO₃, penicillin G, streptomycin (Biochrom AG, Berlin, Germany), and 20% fetal bovine serum (PAA Clone, Cölbe, Germany). After reaching confluence, cells were split using trypsin and then cultured on thin (diameter=15 mm) glass coverslips. Glass coverslips were placed in Petri dishes filled with culture medium. EA.hy926 formed confluent monolayers within 72 h (at 37°C, 5% CO₂). Twenty-four hours prior to the unbinding force measurements, cells were cultured in serum-free medium. Serum deprivation was assumed to upregulate any putative plasma membrane aldosterone receptor sites and thus to increase the probability for their detection on the cell surface.

Chemicals

Aldosterone (*d*-aldosterone, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in ethanol (1 mM stock solution, stored at 4°C for 2 weeks). Final concentration added to the bath solution was 100 nM, aiming to block the putative aldosterone receptor sites. Spironolactone (ICN Biochemicals GmbH, Eschwege, Germany) was dissolved in ethanol (1 mM stock solution) and added to the bath solution at a final concentration of 100 nM. Dexamethasone (water soluble, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in water (1 mM stock solution, stored at -20°C). Final concentration in the bath solution was 100 nM.

Reverse transcription polymerase chain reaction analysis of the classical mineralocorticoid receptor

Total RNA was extracted from EA.hy926 following the standard protocol of RNeasy Mini kit plus (Qiagen, Hilden, Germany). In this procedure, the genomic DNA contamination was effectively removed with a gDNA Eliminator spin column, so that additional DNase digestion was not necessary. Afterwards, the RNA was eluted in 30 µl RNase-free water. RNA was quantified and its quality assessed by spectrophotometry. About 1 µg of total RNA was used to generate first-strand cDNA by using the SuperScript III reverse transcriptase kit (Invitrogen) and oligo (dT) primer. A 900-bp fragment of human mineralocorticoid receptor was amplified by polymerase chain reaction (PCR) using primers 5'-TCAAGTCCGTTAAGTAGCAT-3' and 5'-TTTCCATGACTCCACTAAAG-3'. The reaction mixture consisted of 2 µl cDNA, 1.25 U *Taq* DNA polymerase (Invitrogen), 200 nM of each primer, 200 µM deoxyribonucleotide triphosphate, 10 mM KCl, 10 mM (NH₄)SO₄, 20 mM Tris-HCl pH 8.8, 2 mM MgSO₄, and 0.1% Triton X-100 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 (59°C, 1 min), and synthesis (72°C, 1 min 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 1% agarose gels in 0.5× TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM ethylenediamine tetraacetic acid), stained in 1× SYBR Gold (Molecular Probes, Leiden, Netherlands) in 0.5× TBE, and photographed on a UV transilluminator (Molecular Imager, Biorad, München, Germany).

Conjugation of aldosterone to AFM tips

In contrast to single protein–protein force measurements where, e.g., glutathione-*S*-transferase-fused proteins can be attached to AFM cantilevers [37], aiming to perform force measurements with a steroid-functionalized AFM tip required a different experimental strategy. AFM tips (Si₃N₄, Veeco, Santa Barbara, CA, USA) were amino-functionalized as described before, using the room temperature method for reaction with ethanolamine hydrochloride [20]. The aminofunctionalized tip (tip-NH₂) was incubated for 2 h in 0.5 ml of chloroform containing 2 mg/ml of α -([fluoren-9-yl-methoxycarbonyl]aminopropyl)- ω -{2-[4-(*N*-succinimidyl-oxy-carbonyl)butanoyl-amino]propyl}-poly(oxyethylen)-800 (Fmoc-NH-PEG-CO-NHS, synthesis will be published elsewhere) and 0.5% (*v/v*) of triethylamine to obtain tip-NH-CO-PEG-NH-Fmoc. After rinsing with chloroform, the tip was stored under argon for less than 5 days. The Fmoc-protecting group was removed by immersion in a solution of *N,N*-dimethylformamide/piperidine (4:1, *v/v*) for 2 min and rinsed in dimethylformamide (DMF) and chloroform, thereby obtaining tip-NH-CO-PEG-NH₂. Aldosterone was coupled to the terminal amine by incubating the tips for 2 h in a mixture of aldosterone-COOH, *N,N,N',N'*-tetramethyl(succinimidyl)uronium tetrafluoroborate (for in situ activation of the COOH group in aldosterone), DMF, and *N,N*-diisopropyl-*N*-ethylamine. The tips were then washed in DMF and ethanol, stored under argon, and used within the next 14 days.

Unbinding force measurements

Measurements were performed on living cells at 37°C using a feedback-controlled heating device mounted on an AFM MultiMode® (Veeco, Mannheim, Germany). The cells were bathed in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered solution (composition in mM: 135 NaCl; 5 KCl; 1 MgCl₂; 1 CaCl₂; 10 HEPES; pH 7.4). For the detection of aldosterone interaction sites on the cell surface, force–distance cycles were performed using aldo-

sterone-functionalized cantilevers (metal-to-ligand charge transfer-contact microlevers, nominal spring constants: 0.01 to 0.03 N/m; Veeco, Mannheim, Germany). Spring constants of the cantilevers were determined using the thermal noise method, and analysis of interaction forces was performed using SPIP software (Image Metrology A/S, Horsholm, Denmark).

Results

In a first step, we tested whether the EA.hy926 cells express the classic intracellular mineralocorticoid receptor. As indicated in Fig. 1, reverse transcription polymerase chain reaction (RT-PCR) clearly shows that mRNA of the mineralocorticoid receptor can be detected in this human vascular endothelial cell line. Figure 2 shows schematically how aldosterone is tethered to the AFM tip (Fig. 2a) and the experimental concept (Fig. 2b). The high sensitivity of the AFM and the soft cantilever which was used in the experiments made it possible to detect unbinding forces in the pN range. It has been shown that single-molecule receptor–ligand forces are typically in the range between 50 and 300 pN [1, 2, 7, 26]. Unbinding forces between aldosterone, bound covalently with a spacer to the AFM tip, and the endothelial cell surface were determined by vertical approach–retract cycles. The AFM tips were conjugated with aldosterone via distensible tethers at a very dilute surface concentration, so that statistically only a single aldosterone molecule had access to any receptor sites on the cell surface. Figure 3 shows a typical series of experiments. The left part of the figure (Fig. 3a–c) shows force–distance cycles repeated every 20 s in

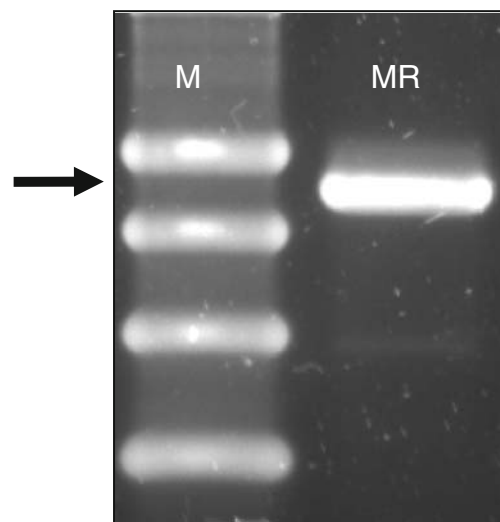
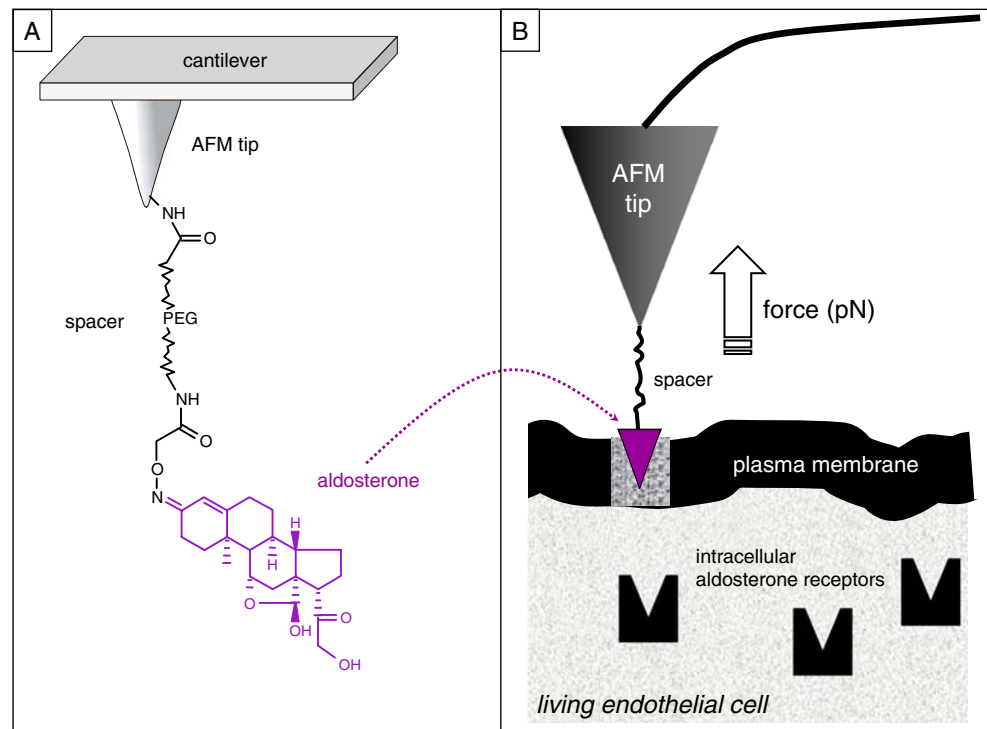


Fig. 1 Detection of the mineralocorticoid receptor (*MR*) in RNA samples extracted from EA.hy926 by using RT-PCR. A 900-bp fragment was amplified with specific primers. *M* smart ladder marker (Eurogentec, Köln, Germany), *arrow* indicates 900 bp

Fig. 2 **a** Schematic of an aldosterone-functionalized AFM tip using a 5-nm long PEG linker as a flexible spacer between tip and hormone. **b** Experimental concept. When a single aldosterone molecule mounted via a spacer to the AFM tip interacts with a membrane receptor, a significant “pull-off force,” termed unbinding force, should be detected. Since the functionalized AFM tip has only access to the outer membrane surface, the classic cytosolic mineralocorticoid receptors cannot interfere with the measurement



the absence of aldosterone in the bath solution (control). First, the tip approaches the cell surface. At some distance from the surface, no bending of the cantilever is visible, and the deflection (in nm) is almost zero. As soon as the cantilever tip

touches the cell surface and is about to indent the membrane, the cantilever bends, which is indicated by an upward deflection. At the same time, aldosterone loaded to the AFM tip binds to the cell surface. The succeeding retraction of the

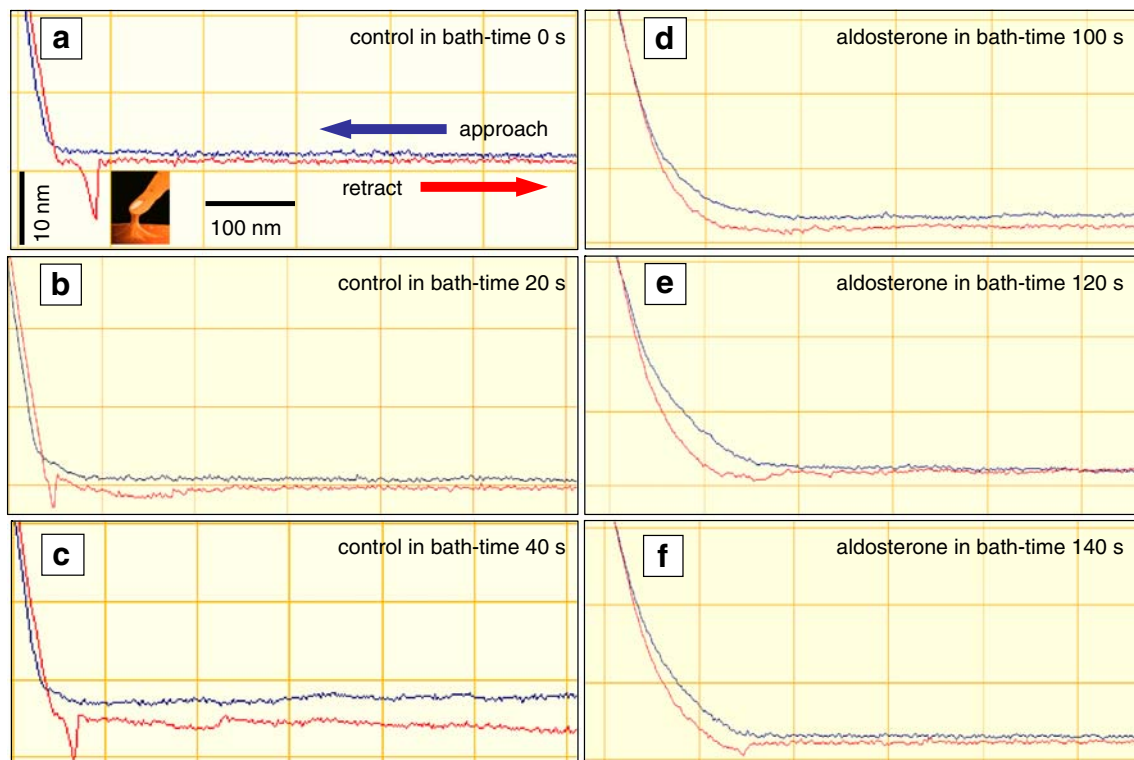


Fig. 3 Representative experiment in a vascular endothelial cell showing six force–distance cycles before (**a–c**) and after (**d–f**) addition of 100 nM aldosterone to the bath solution. The first reliable

measurement after aldosterone addition was made with a delay of 60 s. Measurements were performed on one individual cell

cantilever first results in a relaxation of the indented membrane region followed by a bending of the cantilever in the opposite direction. This is indicated by a downward deflection. The cantilever finally jumps back to its former position when the AFM tip separates from the membrane. The measured sudden jump towards the baseline, deflection Δz (in nm), reflects the unbinding force f according to Hooke's law ($f=k \times \Delta z$, where k is the spring constant of the cantilever). Such force–distance cycles were performed at rates of 0.05 to 0.1 Hz with an AFM tip velocity of 500 nm/s. The upward deflections shown in Fig. 3a–c correspond to unbinding forces of about 60 pN. If the interaction between the aldosterone-conjugated cantilever tip and the plasma membrane were specific, then the addition of aldosterone to the bath solution should saturate any putative aldosterone receptor sites on the cell surface and eliminate tip–membrane interactions. That this is indeed the case is illustrated in Fig. 3d–f. Addition of aldosterone to the bath solution disturbed the force-cycle measurements. For this reason therefore, the first reliable measurement, after aldosterone addition, was made after a delay of 60 s.

Figure 4 shows the histogram of unbinding forces obtained in ten individual endothelial cells. The histogram shows a frequency maximum of about 65 pN. There is another, admittedly small, frequency maximum found at about 125 pN which indicates that, in a small number of experiments, two aldosterone molecules attached to the AFM tip have interacted with two neighboring binding sites at the cell surface. This view is supported by the observation that such large unbinding values often corresponded to sawtooth-like breaks of the interaction sites.

Finally, we measured the binding probabilities under different experimental conditions to test the specificity of the measured interactions between the aldosterone-loaded AFM tip and the cell membrane. For this purpose, we performed force–distance cycles at selected membrane locations which exhibited a positive unbinding signal (>10 pN). The mean data are displayed in Fig. 5. In the absence of aldosterone in the bath solution, the binding probability was $53 \pm 7.2\%$. It decreased to $8 \pm 1.8\%$ when 100 nM aldosterone was added to the bath. In contrast, addition of either 100 nM spironolactone or 100 nM dexamethasone to the bath solution did not significantly influence the binding probability ($57 \pm 8.1\%$ and $50 \pm 8.6\%$, respectively) which indicates that the binding sites on the cell surface are specific for aldosterone and are not related to the classic intracellular receptors.

Addition of aldosterone to the bath solution swells endothelial cells within minutes as reported previously [22, 24, 31]. However, such a cell swelling is not expected to interfere with the unbinding measurements since the distance between AFM tip and cell surface was kept constant during the measurements.

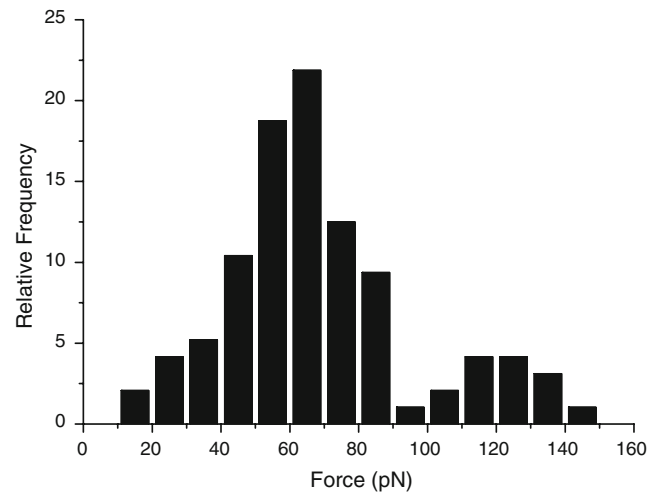


Fig. 4 Frequency distribution of unbinding forces obtained with an aldosterone-functionalized AFM tip on the surface of vascular endothelial cells

Discussion

Until the mid-1980s, the physiological and pathophysiological role of aldosterone had a certain lucidity, namely, that the mineralocorticoid acts on epithelia and binds to

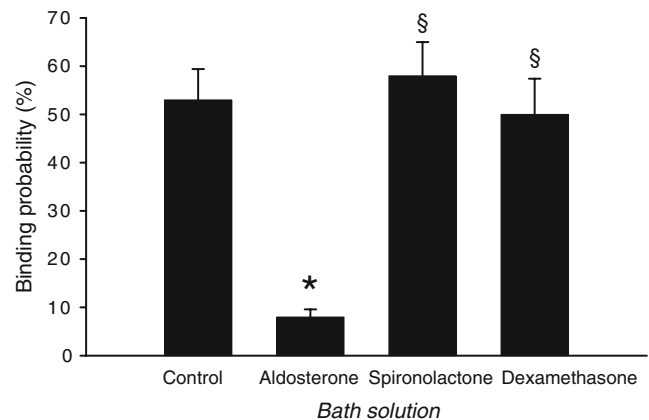


Fig. 5 Binding probabilities of the aldosterone-functionalized AFM tip with the endothelial cell membrane. Either 100 nM aldosterone, 100 nM spironolactone, or 100 nM dexamethasone was added to the bath to test the specificity of the measured interactions. The binding probability between tip and sample was calculated from the number of force–distance cycles with clear unbinding divided by the total number of force–distance cycles (with and without unbinding) multiplied by 100 (%). Each column represents the mean data (\pm SEM) obtained in five cells. About 100 force–distance cycles were performed per cell. Each series was started in control solution at selected membrane sites with significant tip–sample interactions. Then, the respective hormones/inhibitors were added. Addition of aldosterone to the bath solution reduced the binding probability significantly indicated by *asterisk* (paired Student's *t* test: $p < 0.001$; control vs. aldosterone) in contrast to the addition of either spironolactone or dexamethasone which were ineffective indicated by *section sign* (paired Student's *t* test: $p > 0.05$; control vs. spironolactone or dexamethasone)

cytosolic receptors which then enter the nucleus and initiate gene transcription. De novo synthesis followed this genomic action, which led to changes in the function of the target cell. Over the last two decades, it had become increasingly apparent that besides the slow (hours) genomic signaling pathway, there was, in parallel, a nongenomic pathway characterized by an early onset (minutes) and an insensitivity to transcription inhibitors. This early response of aldosterone on target cells that was not only observed in epithelia but also in other types of cells [11, 28–31] could be functionally associated with ion transport mechanisms in the plasma membrane. Serious attempts were made to identify a specific plasma membrane aldosterone receptor, but the final proof for its existence is still missing [9]. Moreover, over the past years, there has been accumulating evidence that the classic cytosolic receptor could account for both genomic and nongenomic responses [10, 14, 15]. Since we and most other researchers had concentrated on this new concept, there was only little interest in an aldosterone receptor in the plasma membrane. Work in our laboratory, however, had indicated that, in the earliest phase of aldosterone action, the classic cytosolic mineralocorticoid receptor is not involved [24]. Endothelial cell swelling which occurred in the very first minute of aldosterone application could not be blocked by the receptor antagonist spironolactone. However, 4 min later, this intracellular receptor blocker turned out to be indeed effective [24]. Since most studies measured the rapid response of aldosterone and its successful inhibition by mineralocorticoid antagonists *after* the first minute, this initial, very early and possibly membrane-associated response to aldosterone was overlooked.

With the manufacture of functionalized AFM tips that can physically interact with the cell membrane in the

nanoscale range, it has become possible to recognize specific sites on artificial surfaces and cells [5, 18]. Applying this technique, VE-cadherin-binding sites on fixed endothelial cells could be identified at a lateral resolution close to 5 nm [4]. In the present study, using living human vascular endothelial cells (EA.hy926 cell line) known to respond to aldosterone [23] and to be capable of expressing the classical intracellular mineralocorticoid receptor (this study), we tried to find out whether specific binding sites for aldosterone were identifiable on the apical cell membranes. A 5-nm polyethylene glycol (PEG) linker was used, covalently bound to the AFM tip at its proximal end and covalently bound to a single aldosterone molecule at its distal end. By choosing appropriate linker concentrations, the amount of attached molecules per AFM tip can be controlled so that, statistically, a single molecule at the very tip of the AFM stylus is available for the interaction with the plasma membrane [20]. We performed force curves at rates of about 0.05 to 0.1 Hz while guiding the functionalized tip to different spots on the cell membrane. In the moment of an unbinding event, the search for a membrane location was stopped and repetitive force curves sampled at this spot. Thus, it was possible to measure the unbinding forces and, at the same time, the binding probability by counting the unbinding events directly from the screen and to analyze unbinding forces from the stored force curves after the experiment. Furthermore, this technical approach made it possible to perform inhibition experiments in a paired fashion, i.e., first an interaction site was identified, and then, the potential inhibitor was added.

Calculations suggested an unbinding force of about 65 pN, a value which is in the range of previous data [1, 2, 7, 26, 32]. Sometimes, there were unbinding forces

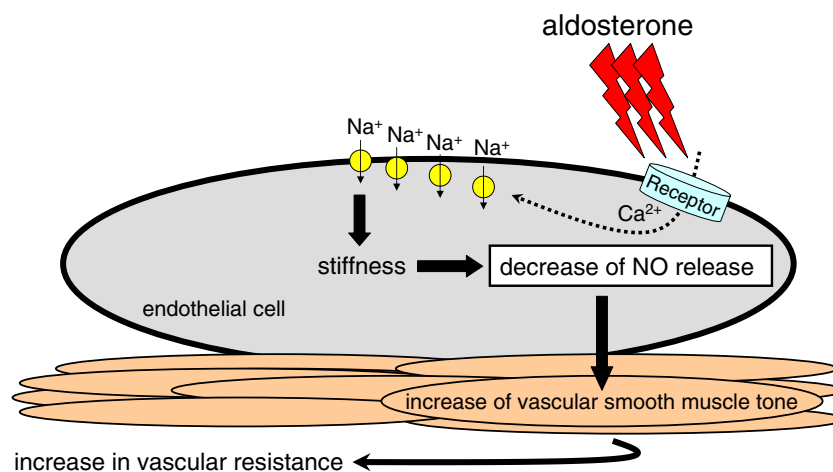


Fig. 6 Schematic model illustrating the potential physiological role of aldosterone receptors in the plasma membrane of vascular endothelial cells. Interaction of aldosterone with its surface receptor leads to Ca²⁺ influx, which triggers the insertion/activation of ENaC in the cell

membrane. Subsequent Na⁺ influx stiffens the endothelial cell and decreases NO release. This could finally increase smooth muscle tone and vascular resistance

with a peak value of about 125 pN indicating that possibly two aldosterone molecules located on the AFM tip had interacted with two membrane receptor sites. When aldosterone was added in an excess concentration to the bath, the tip–cell interaction was inhibited. Although unbinding forces should theoretically collapse to zero (complete block), some remnant small unbinding forces with high variability were sometimes found. A possible explanation could be unspecific hydrophobic interaction between an aldosterone molecule bound to the AFM tip and the one bound to the specific membrane site after addition of the hormone to the bath solution.

Our results cannot distinguish whether the aldosterone receptor sites are located directly in the plasma membrane or somewhere in the glycocalyx that forms an apical layer of several 100 nm on the cell surface. However, the receptor sites that we describe in this paper are apparently specific for aldosterone in contrast to locations in cell-free areas (e.g., gaps between cells which can be found even in confluent monolayers). There, we sometimes observed highly variable unspecific adhesion forces which could not be influenced by the addition of aldosterone to the bath solution (data not shown).

The measurements do not allow estimation of the number of aldosterone receptor sites per cell. The way how the experiments were performed was to move the aldosterone-functionalized AFM tip in the micrometer range across the cell surface, allowing to perform force–distance cycles at about ten different locations per cell. In average, we found an aldosterone-specific receptor site (i.e., a force–distance cycle that could be blocked by the addition of aldosterone to the bath solution) in every third cell. This means that, out of thirty trials to detect a specific aldosterone receptor site, one was successful.

Addition of an excess supply of aldosterone to the bath solution was used to saturate any aldosterone receptor sites and to demonstrate the specificity of hormone binding. Then, the rupture of the binding force following aldosterone addition to the bath solution could be interpreted as the competitive inhibition between free aldosterone added to the bath and aldosterone linked to the AFM tip or as a rapid internalization of the aldosterone receptor. So far, we cannot distinguish between these two possibilities. Since we observed slow and often incomplete reversibility (i.e., recovery of the unbinding force after removal of free aldosterone from the bath solution), we currently favor the view of rapid internalization.

It is interesting to note that neither spironolactone nor dexamethasone influenced the binding between aldosterone and its interaction site on the membrane. This excludes the classic mineralocorticoid and glucocorticoid receptors as potential candidates for this interaction. It strongly supports the view that specific aldosterone interaction sites exist on

the endothelial cell surface structurally and functionally distinct from the cytosolic receptors.

Physiological perspectives

Figure 6 shows a schematic model illustrating the potential relevance of aldosterone surface receptors. According to this model, aldosterone interacts with a plasma membrane receptor site that mediates Ca^{2+} influx from the extracellular space into the cell cytosol. This view is supported by a previous report in porcine endothelial cells where a rise of Ca^{2+} in the subplasmalemmal space was detected only 1 to 5 min after aldosterone exposure [30]. The increase in subplasmalemmal Ca^{2+} could trigger the insertion of epithelial sodium channels (ENaC) stored in the subapical space. This argument is based on a report that shows the appearance of an amiloride-inhibitable cell swelling a few minutes after aldosterone exposure [24]. ENaC-mediated sodium influx is known to stiffen endothelial cells and to reduce nitric oxide release [23].

In conclusion, the signaling cascade described in Fig. 6 is supposed to precede the classic genomic response and thus most likely participates in the regulation of blood pressure.

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References

- Baumgartner W, Hinterdorfer P, Ness W, Raab A, Vestweber D, Schindler H, Drenckhahn D (2000) Cadherin interaction probed by atomic force microscopy. *Proc Natl Acad Sci USA* 97:4005–4010
- Bucior I, Scheuring S, Engel A, Burger MM (2004) Carbohydrate–carbohydrate interaction provides adhesion force and specificity for cellular recognition. *J Cell Biol* 165:529–537
- Christ M, Wehling M (1999) Rapid actions of aldosterone: lymphocytes, vascular smooth muscle and endothelial cells. *Steroids* 64:35–41
- Chitchevlova LA, Atalar F, Ozbek U, Wildling L, Ebner A, Hinterdorfer P (2008) Localization of the ergotoxin-1 receptors on the voltage sensing domain of hERG K(+) channel by AFM recognition imaging. *Pflugers Arch* 456:247–254
- Duffrene YF, Hinterdorfer P (2008) Recent progress in AFM molecular recognition studies. *Pflugers Arch* 456:237–245
- Edgell CJ, Haizlip JE, Bagnell CR, Pakenham JP, Harrison P, Wilbourn B, Madden VJ (1990) Endothelium specific Weibel-Palade bodies in a continuous human cell line, EA.hy926. *In Vitro Cell Dev Biol* 26:1167–1172
- Florin EL, Moy VT, Gaub HE (1994) Adhesion forces between individual ligand–receptor pairs. *Science* 264:415–417
- Fuller PJ, Young MJ (2005) Mechanisms of mineralocorticoid action. *Hypertension* 46:1227–1235

9. Funder JW (2005) The nongenomic actions of aldosterone. *Endocr Rev* 26:313–321
10. Funder JW (2006) Minireview: aldosterone and the cardiovascular system: genomic and nongenomic effects. *Endocrinology* 147:5564–5567
11. Gekle M, Golenhofen N, Oberleithner H, Silbernagl S (1996) Rapid activation of Na^+/H^+ exchange by aldosterone in renal epithelial cells requires Ca^{2+} and stimulation of a plasma membrane proton conductance. *Proc Natl Acad Sci USA* 93:10500–10504
12. Giocondi MC, Seantier B, Dosset P, Milhiet PE, Le Grimellec C (2008) Characterizing the interactions between GPI-anchored alkaline phosphatases and membrane domains by AFM. *Pflügers Arch* 456:179–188
13. Goerge T, Niemeyer A, Rogge P, Ossig R, Oberleithner H, Schneider SW (2002) Secretion pores in human endothelial cells during acute hypoxia. *J Membr Biol* 187:203–211
14. Grossmann C, Benesic A, Krug AW, Freudinger R, Mildnerberger S, Gassner B, Gekle M (2005) Human mineralocorticoid receptor expression renders cells responsive for nongenotropic aldosterone actions. *Mol Endocrinol* 19:1697–1710
15. Grossmann C, Freudinger R, Mildnerberger S, Husse B, Gekle M (2008) EF domains are sufficient for nongenomic mineralocorticoid receptor actions. *J Biol Chem* 283:7109–7116
16. Harvey BJ, Ehrenfeld J (1988) Role of Na^+/H^+ exchange in the control of intracellular pH and cell membrane conductances in frog skin epithelium. *J Gen Physiol* 92:793–810
17. Helenius J, Heisenberg CP, Gaub HE, Muller DJ (2008) Single-cell force spectroscopy. *J Cell Sci* 121:1785–1791
18. Hinterdorfer P, Dufrene YF (2006) Detection and localization of single molecular recognition events using atomic force microscopy. *Nat Methods* 3:347–355
19. Jaffe EA, Nachman RL, Becker CG, Minick CR (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 52:2745–2756
20. Kamruzzahan AS, Ebner A, Wildling L, Kienberger F, Riener CK, Hahn CD, Pollheimer PD, Winklehner P, Holz M, Lackner B, Schorkl DM, Hinterdorfer P, Gruber HJ (2006) Antibody linking to atomic force microscope tips via disulfide bond formation. *Bioconjug Chem* 17:1473–1481
21. Oberleithner H (2004) Unorthodox sites and modes of aldosterone action. *News Physiol Sci* 19:51–54
22. Oberleithner H (2007) Is the vascular endothelium under the control of aldosterone? Facts and hypothesis. *Pflügers Arch* 454:187–193
23. Oberleithner H, Riethmuller C, Schillers H, MacGregor GA, de Wardener HE, Hausberg M (2007) Plasma sodium stiffens vascular endothelium and reduces nitric oxide release. *Proc Natl Acad Sci USA* 104:16281–16286
24. Oberleithner H, Schneider SW, Albermann L, Hillebrand U, Ludwig T, Riethmuller C, Shahin V, Schafer C, Schillers H (2003) Endothelial cell swelling by aldosterone. *J Membr Biol* 196:163–172
25. Oberleithner H, Weigt M, Westphale H-J, Wang W (1987) Aldosterone activates Na^+/H^+ exchange and raises cytoplasmic pH in target cells of the amphibian kidney. *Proc Natl Acad Sci USA* 84:1464–1468
26. Pfister G, Stroh CM, Perschinka H, Kind M, Knoflach M, Hinterdorfer P, Wick G (2005) Detection of HSP60 on the membrane surface of stressed human endothelial cells by atomic force and confocal microscopy. *J Cell Sci* 118:1587–1594
27. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 307:1625–1630
28. Rocha R, Funder JW (2002) The pathophysiology of aldosterone in the cardiovascular system. *Ann NY Acad Sci* 970:89–100
29. Schmidt BM, Sammer U, Fleischmann I, Schlaich M, Delles C, Schmieder RE (2006) Rapid nongenomic effects of aldosterone on the renal vasculature in humans. *Hypertension* 47:650–655
30. Schneider M, Ulsenheimer A, Christ M, Wehling M (1997) Nongenomic effects of aldosterone on intracellular calcium in porcine endothelial cells. *Am J Physiol* 272:E616–E620
31. Schneider SW, Yano Y, Sumpio BE, Jena BP, Geibel JP, Gekle M, Oberleithner H (1997) Rapid aldosterone-induced cell volume increase of endothelial cells measured by the atomic force microscope. *Cell Biol Int* 21:759–768
32. Sotres J, Lostao A, Wildling L, Ebner A, Gomez-Moreno C, Gruber HJ, Hinterdorfer P, Baro AM (2008) Unbinding molecular recognition force maps of localized single receptor molecules by atomic force microscopy. *Chemphyschem* 9:590–599
33. Wehling M, Christ M, Theisen K (1991) High affinity aldosterone binding to plasma membrane rich fractions from mononuclear leukocytes: is there a membrane receptor for mineralocorticoids. *Biochem Biophys Res Commun* 181:1306–1312
34. Wehling M, Christ M, Theisen K (1992) Membrane receptors for aldosterone: a novel pathway for mineralocorticoid action. *Am J Physiol* 263:E974–E979
35. Wehling M, Eisen Ch, Aktas J, Christ M, Theisen K (1992) Photoaffinity labeling of plasma membrane receptors for aldosterone from mononuclear leukocytes. *Biochem Biophys Res Commun* 189:1424–1428
36. Wehling M, Kasmayr J, Theisen K (1989) Fast effects of aldosterone on electrolytes in human lymphocytes are mediated by the sodium-proton-exchanger of the cell membrane. *Biochem Biophys Res Commun* 164:961–967
37. Yoshimura SH, Takahashi H, Otsuka S, Takeyasu K (2006) Development of glutathione-coupled cantilever for the single-molecule force measurement by scanning force microscopy. *FEBS Lett* 580:3961–3965