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Topical Review

Nuclear Envelope: Nanoarray Responsive to Aldosterone

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Abstract. Signalling between cytosol and nucleus is mediated by nuclear pores. These supramolecular complexes represent intelligent nanomachines regulated by a wide spectrum of factors. Among them, steroid hormones specifically interact with the pores and thus modify ion conductivity and macromolecule permeability of the nuclear envelope. In response to aldosterone the pores undergo dramatic changes in conformation, changes that depend on the nature of the transported cargo. Such changes can be imaged at the nanometer scale by using atomic force microscopy. Furthermore, steroid-induced macromolecule transport across the nuclear envelope causes osmotic water movements and nuclear swelling. Drugs that interact with intracellular steroid receptors (spironolactone) or with plasma membrane sodium channels (amiloride) inhibit swelling. Steroid hormone action is blocked when nuclear volume changes are prevented. This is shown in frog oocytes and human endothelial cells. In conclusion, nuclear pores serve as steroid-sensitive gates that determine nuclear activity.

Introduction

In 1994 a paper was published that contained a rather unusual oberservation made with a rather unusual technique (Oberleithner et al., l994). The unusual observation was the increase in number of nuclear pore complexes in nuclear envelopes of kidney cells in response to aldosterone, and the unusual technique applied in this study was atomic force microscopy (AFM). At that time, aldosterone had been considered a hormone that controlled fluid and electrolyte balance in kidney through regulation of plasma membrane ion channels and transporters, but virtually nothing was known about its interaction with the nuclear barrier. Possibly, those AFM experiments were born by the desperate desire of a few renal physiologists who wanted to apply a new nanotechnique, originally developed by physicists working in the material sciences (Binnig & Quate, 1986), on a biological membrane with some relevance for kidney function. In the meantime 10 years have passed. During this time, the understanding of aldosterone physiology underwent a dazzling metamorphosis in terms of site and mode of action of this hormone (Oberleithner, 2004), AFM developed into a useful tool in the biological sciences (Roco, 2003), and finally, the nuclear envelope became an extensively explored membrane system that selectively passes signals from outside into the nucleus (Gerasimenko et al., 2003). In this short review I will focus on recent developments in this field.

Atomic Force Microscopy

The atomic force microscope works by moving a probe back and forth across a surface and recording features as the probe encounters them. AFM produces images that are not compromised by the limitations of the wavelengths of the various types of electromagnetic radiation. This means that very high resolution (below 1 nm) can be obtained. Biological work using the AFM has shown considerable growth since its invention in 1986 (Binnig & Quate, 1986). The AFM probe (i.e., the AFM tip that scans the sample) is typically made from a pyramidal crystal of silicon nitride deposited onto a gold-coated, flexible cantilever. A sample (e.g., the nuclear envelope) is prepared on a flat substrate (i.e., glass or mica) and moved so that it makes contact with the probe. The sample is then moved in a raster pattern and the cantilever carrying the probe is deflected vertically as features in the sample move under it. The movement is controlled by a series of piezoelectric drivers and the control is such that the probe can be positioned in either horizontal ‘x’ or ‘y’ dimensions or in the

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vertical 'z' dimension very accurately. A low-powered laser is focused onto the cantilever and is reflected onto a series of photomultiplier detector elements. As a result, when the probe scans across a surface and meets some sort of obstacle, the cantilever is deflected, changing the reflected angle of the laser, and therefore affecting the signal detected by the photomultipliers. The signal is fed into a computer, which then constructs a three-dimensional image from the information received. The principle of the AFM is therefore quite simple, and its implementation is dependent on the availability of suitable sensitive photomultipliers and piezo control mechanisms. More about the different modes of recording high resolution AFM images can be found in a recent review (Henderson & Oberleithner, 2000).

Aldosterone

The traditional concept of aldosterone action is the binding of the ligand to classic mineralocorticoid receptors (MRs) in target cells of kidney, colon, salivary and sweat glands. It is followed by a genomic response and an appropriate change in physiological function of the target cell. Over the past years, the endocrine properties of aldosterone have taken on a broader perspective involving non-classic actions in non-epithelial cells found in non-classic target tissues (Epstein, 2001). At least four new perspectives shape up, related to aldosterone action. The fact that the MRs play a crucial role in the function of a broad variety of cell types should not lead to an underestimation of the potential role of rapid (apparently non-genomic) effects elicited by this hormone.

(i) Many more different cell types than previously thought express the mineralocorticoid receptor. Besides the above mentioned classic aldosterone-responsive tissues, MR are found also in endothelial cells, cardiac myocytes, fibroblasts, vascular smooth muscle cells and many others. In parallel, evidence for distinct changes in cellular functions elicited by aldosterone and blocked by specific inhibitors is accumulating in the literature (Delyani, 2000).

(ii) Aldosterone exerts rapid effects, non-genomic in nature (Falkenstein et al., 2000). These effects have a rapid time course (seconds to minutes) and are not inhibited by actinomycin D. They have been considered to reflect aldosterone acting via a high-affinity membrane receptor, distinct from the classic intracellular MR for two reasons. One is that the rapid effects have not been inhibited by spironolactone and another is that glucocorticoids do not mimic the effects of aldosterone at nanomolar concentrations.

(iii) Rapid, non-genomic effects of aldosterone may also reflect actions via the classic intracellular MR. Such a scenario can be derived from experiments in vascular smooth muscle cells (Alzamora, Michea & Matusic, 2000), cardiac myocytes (Mihailidou et al., 2000) and vascular endothelial cells (Golestaneh et al., 2001) discussed in more detail in a recent review (Funder, 2001).

(iv) Aldosterone action may not even depend upon aldosterone secreted classically from the suprarenal glands but may be triggered by aldosterone released directly at the site of action. Indeed, recent experiments in endothelial cells and cardiomyocytes show that the complete cascade, the renin-angiotensin-aldosterone system (RAAS), including MR, can be localized within the heart (Hatakeyama et al., 1994; Silvestre et al., 1998). This suggests that observations like mineralocorticoid-induced vascular remodeling and hypertension may stem from the paracrine and/or autocrine action of locally produced aldosterone released in response to angiotensin II derived from the vascular wall. In other words, the vascular bed is endowed with all of the cellular effectors that had been believed to exist exclusively in selected organs such as the kidney.

Xenopus laevis Oocyte

Insight into aldosterone action at the subcellular level was recently gained by investigating a rather non-classic target cell, namely the *Xenopus laevis* oocyte, with atomic force microscopy and electrical methods. The biological model, the technical approaches and the major experimental results have been published in detail previously (Danker et al., 1999; Shahin et al., 2001; Schafer et al., 2002). The reason why oocytes became a suitable model for studying aldosterone action at the molecular level was the following: Stage-VI *Xenopus laevis* oocytes are inactive in terms of transcription but can be activated by exogenous stimuli (Golden, Schafer & Rosbash, 1980; Leonard & La Marca, 1975). They respond to aldosterone by triggering a fast signal cascade involving the cell nucleus. Some molecules of this signal cascade interact with the nuclear barrier and thus can be studied at the single molecule level.

Nuclear Pores

In eukaryotic cells two concentric membranes in parallel separate the nucleus from the cytoplasm. This so-called nuclear envelope is punctured by nuclear pore complexes (NPCs) that serve as regulated pathways for macromolecules entering and exiting

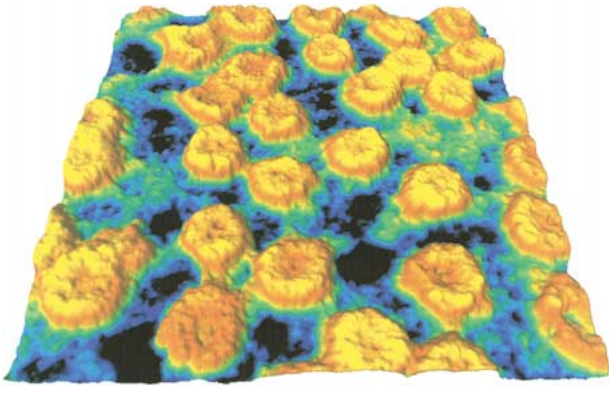

Fig. 1. Nuclear envelope of *Xenopus laevis* oocyte, a 'biological nanoarray', imaged with atomic force microscopy. The area is about 600 × 600 nm. The image height (*z*-axis) is 15 nm.Nuclear pore complexes are the yellow structures. The lipid bilayer membrane visible between the pores is depicted as more or less dark-blue (after Mazzanti et al., 2001).

the nuclear compartment (Fig. 1) Transport across NPCs (each NPC has a molecular mass of 120 MD) occurs through central channels. Such import and export of macromolecules through individual NPCs could be elicited in *Xenopus laevis* oocytes by injection of aldosterone and visualized with AFM.

Flags on Pores

Mineralocorticoid receptors, usually located in the cytosol in an inactive state, move rapidly towards the cell nucleus upon activation. Two minutes after aldosterone injection into a *Xenopus laevis* oocyte a homogeneous population of macromolecules was found attached to the cytoplasmic surface of NPCs. These macromolecules were termed 'flags' since they appeared 'shaky' when scanned. Such a 'shaky' appearance indicated a rather loose interaction of the macromolecule with the NPC (Fig. 2). Few minutes later, the flags had disappeared (Schafer et al., 2002). Flags may resemble the aldosterone receptors. From the volume of the individual macromolecules, measured by AFM (Henderson et al., 1996; Schneider et al., 1998; Pietrasanta et al., 1999; Berge et al., 2000), a molecular mass of about 100 kD per molecule could be estimated. This is a fair match of the molecular mass of a monomeric MR (Arriza et al., 1987). Nevertheless, AFM can only estimate the volume with considerable scatter and there is yet no proof for any specificity. The finding that the putative receptors disappeared a few minutes after aldosterone injection was probably caused by transport through the NPC central channels into the nucleoplasm.

Plugs in Pores

Twenty minutes after hormone injection large plugs (≈1 MD) appeared in the central NPC channels (Schafer et al., 2002). They may be ribonucleoproteins exiting the nucleus (Fig. 3). If plugs were located close to the surface of individual NPCs they could be detected by AFM. They had large dimensions (about 40 nm in diameter and 8 nm in height) and could even be harvested by the AFM stylus. Indications that plugs could be the first products of the genomic response came from the molecular mass that fairly matched the mass of ribonucleoproteins (Sperling et al., 1997). Further support for this theory could be derived from the delayed response, i.e., plug formation 20 minutes after hormone injection.

Nuclear Pore Channels

Aldosterone transiently changed the electrical properties of the nuclear envelope (Schafer et al., 2002). The observed changes correlated with the morphological changes of the NPCs observed with AFM. Electrical resistance measurements in isolated nuclei revealed transient electrical nuclear envelope resistance peaks following aldosterone treatment, an early (2 min) peak and a late (20 min) peak. Electrical peaks reflect macromolecule interaction with NPCs. As worked out by elegant studies of Bustamante et al. (Bustamante, Hanover & Liepins, 1995), macromolecules can serve as dielectric insulators when plugging the central channels. Thus, the early peak in nuclear envelope electrical resistance induced by aldosterone was not due to plugging of the central channel with putative MR but rather due to blockage of the so-called peripheral channels of NPCs. Evidence for such small peripheral channels located in the NPC rings surrounding the large central channel came from electron microscopy (Hinshaw, Carregher & Milligan, 1992). We recently (Shahin et al., 2001) confirmed the existence of peripheral channels with AFM (Fig. 4). The fact that the 100 kD molecules (flags) decorated the cytoplasmic rings explained the early peak in electrical resistance. The flags (presumably aldosterone receptors) physically blocked the small peripheral channels and prevented ion fluxes that finally determined nuclear envelope electrical resistance. The late peak was probably caused by the plugs that exited the central channels. Based on these experiments, a model was proposed in which the central channel (60 nm in length) is more or less always congested with macromolecules that prevent ion flux. The 'real' diffusional ion permeability, however, is based on the function of small peripheral channels that can be in the open configuration (resting pores that allow ion equilibrium between cytosol and nucleoplasm), in the 'blocked' configuration (macromolecules on pore surface occupying the entrances of the small peripheral channels and causing a transient ion disequilibrium between cytosol and nucleoplasm) or in the 'squeezed' configuration (large

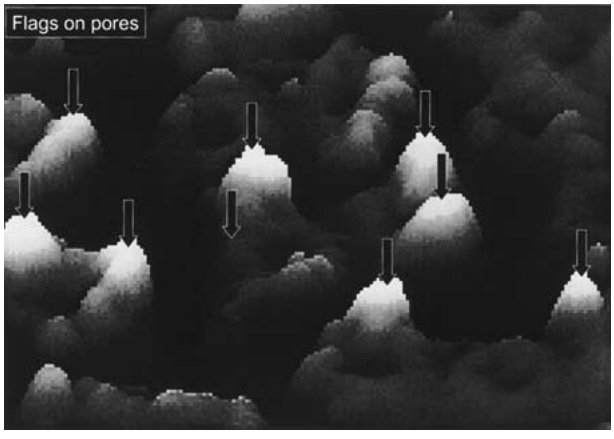

Fig. 2. AFM imaging of 'flags' on nuclear pores. Putative mineralocorticoid receptors (flags are indicated by arrows) attached to the rims of nuclear pores (ring-like structures with central channels) two minutes after aldosterone stimulation. Individual pores are about 80 to 100 nm. The image height (z-axis) is 10 nm. Flags (about 8 nm in height) disappear a minute later, probably due to nuclear import (after Schafer et al., 2002).

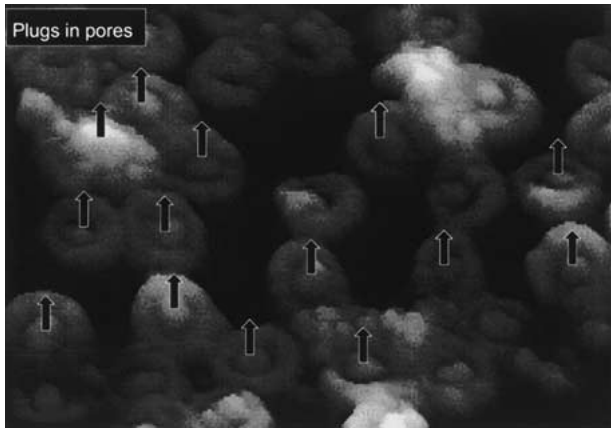

Fig. 3. AFM imaging of 'plugs' in nuclear pores. Putative ribonucleoproteins (plugs are indicated by arrows that point in export direction) located in the central channels of nuclear pores 20 minutes after aldosterone stimulation. Individual pores are dilated to outer diameters of about 120 to 140 nm. The image height (z-axis) is 10 nm. Plugs disappear a few minutes later, probably due to export into the cytosol (after Schafer et al., 2002).

ribonucleoproteins exiting the central channels causing a transient ion disequilibrium between cytosol and nucleoplasm similar as mentioned above (for details *see* Mazzanti, Bustamante & Oberleithner, 2001)

Spironolactone

Spironolactone is an aldosterone antagonist that competes for the same binding site on the MR. Upon spironolactone binding, the release of heat shock protein hsp 90 is facilitated leaving behind a destabilized form of MR (Couette et al., 1996). Surprisingly, structural as well as functional studies revealed that spironolactone interfered already with MR vectorial transport, directed from cytosol to nucleus. With AFM, putative MRs (flags) could not be detected on the NPC surface if spironolactone was coinjected with aldosterone (Schafer et al., 2002). At the same time, the early electrical peak was missing. Both AFM and electrical data suggest that the putative MRs do not move towards the nuclear envelope if bound to spironolactone. Data were in agreement with studies using confocal fluorescence microscopy to trace the translocation of MRs from the cytosol into the cell nucleus (Fejes-Toth, Pearce & Naray-Fejes-Toth, 1998). The researchers convincingly showed that spironolactone strongly attenuated intranuclear MR accumulation. It was not surprising that spironolactone also inhibited the late response (i.e., the late electrical resistance peak and the plug appearance in the NPC central channels). This was the logic consequence, since the blocker already interfered with the pre-genomic signal cascade.

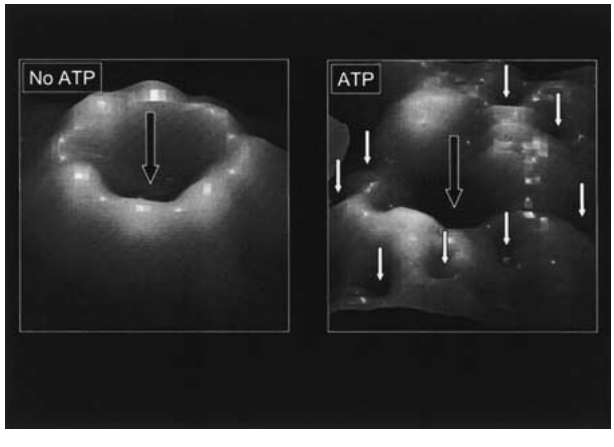

Fig. 4. Effect of ATP (and calcium) on the conformation of individual nuclear pore complexes of *Xenopus laevis* oocyte. ATP (plus calcium) induces the formation of so-called peripheral channels (*small arrows*) circularly arranged around the central channel (*large arrow*). Each AFM image is about 100 nm in width. The image height (z-axis) is 10 nm (after Shahin et al., 2001).

Actinomycin D

Actinomycin D is known to inhibit transcriptional processes in the cell nucleus triggered by steroid hormones. Application of this drug in aldosterone-injected oocytes prevented the late aldosterone response, i.e., the late electrical resistance peak of the nuclear envelope and the appearance of plugs (ribonucleoproteins) in NPC central channels (Oberleithner et al., 2000). This observation strongly indicated that NPC plugs originate from transcriptional processes elicited by aldosterone. In contrast, Actinomycin D was ineffective at blocking the early response,

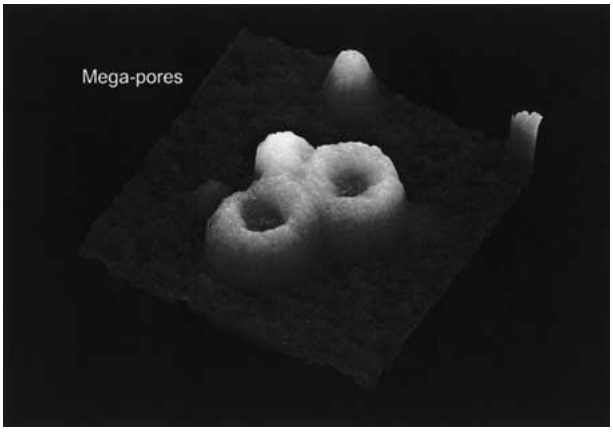

Fig. 5. AFM images of so-called mega-pores found in the nuclear envelope of *Xenopus laevis* oocyte 10 minutes after aldosterone injection. The mega-pores have outer diameters in the range of 1000 nm and a height of about 150 nm. They are composed of close to a hundred individual nuclear pores (visible in the flat part of this image). The estimated life-time of these large pores is in the range of a few minutes. They are candidates for mediating a large transient macromolecule permeability of the nuclear envelope (unpublished observation in the author's laboratory).

which confirms that the early electrical peak usually observable after aldosterone injection was pre-genomic in nature. The aldosterone-induced transcriptional process in the oocyte nucleus was found to have an electrical correlate. Electrical resistance of the nuclear envelope transiently decreased within a narrow time segment (Schafer et al., 2002). This electrical leak of the nuclear envelope was accompanied by a passive permeability leak for small macromolecules (Buchholz et al., 2004). The leak pathway induced by aldosterone lasted only minutes. Evidence for the transient formation of so-called 'mega-pores' could be derived from recent AFM experiments on nuclear envelopes studied right at the time when aldosterone-induced transcription processes occurred in the nucleus (unpublished observation in the author's laboratory). Mega-pores resembled suprastructures in the nuclear envelope, made up by a large number of individual NPCs arranged around a 'mega-channel' (Fig. 5).

Table 1 summarizes the changes in nuclear envelope structure and function in response to aldosterone.

Intracellular Calcium

Rise in intracellular free calcium is the typical early response of a target cell when exposed to aldosterone (Gekle et al., 1996; Schneider et al., 1997a; Harvey & Higgins, 2000). It occurs a few seconds after hormone application and is usually transient in nature. Beyond doubt, it is a pre-genomic cellular event that still lacks an explanation concerning its physiological relevance. Recent data indicate a potentially important role for intracellular free calcium (Schafer et al., 2003). When EGTA was coinjected with aldosterone, the early and the late electrical peaks were missing. Obviously, free calcium is necessary for an appropriate aldosterone response. Since the early electrical resistance increase reflects MR docking to NPCs, it was assumed that MR docking is missing under low-calcium conditions. The mechanism by which calcium ions could interfere with the pre-genomic signalling pathway remains obscure. Calcium could be neccessary for hormone-receptor interaction, for vectorial movement of activated receptors towards the nuclear envelope and for docking of hormone receptors to NPCs. MRs are known to be associated with cytoskeletal structures (Jalaguier et al., 1996; Golestaneh et al., 2001). According to the literature, aldosterone abolishes such interactions and obviously allows MRs to move on. This could be a crucial step in MR translocation into the cell nucleus. Calcium ions are supposed to play a role in this scenario. It is concluded that the intracellular calcium increase observed in response to aldosterone enables MRs to travel to NPCs. Then, MRs are translocated into the nucleoplasm. Only in presence of appropriate concentrations of ionized calcium can a cell properly respond to the steroid.

Endothelium

Some years ago it was observed in living aortic endothelial cells that aldosterone transiently increased cell volume (Schneider et al., 1997b). The aldosterone response occurred in minutes and could be inhibited by amiloride. Since AFM was applied in this previous study it was possible to analyze the 3-D morphology of the adherent endothelial cells together with cell volume. Although AFM images of living cells have only poor resolution it became obvious in a later analysis (Oberleithner et al., 2000) that the volume change mainly occurred in the cell nucleus. Based on this data it was postulated that volume "cycles" between intracellular compartments are induced by aldosterone.

Nuclear Volume Cycling

In order to shift volume from the cytosol to the nucleoplasm, osmotic driving forces are necessary. Macromolecule movements occur between cytosol and nucleoplasm in response to steroid hormones. Most likely, changes in water balance are mediated by receptor import into the cell nucleus and export of transcribed mRNA into the cytosol. Such macromolecule shifts together with condensation/decondensation processes of the chromatin induced by

Table 1. Changes in nuclear envelope structure and function in response to aldosterone

Parameter	0–2 min After aldosterone	8–15 min After aldosterone	18–24 min After aldosterone
Nuclear envelope electrical resistance	Increased	Decreased	Increased
Nuclear pore configuration	Flags on pores	Pores free	Plugs in pores
Nuclear envelope dextran permeability	Unchanged	Strongly increased	Unchanged
Interpretation (Schafer et al., 2002; Oberleithner et al., 2003)	Mineralocorticoid receptor docking on nuclear pore surface blocks small peripheral channels	Transcription processes going on in the cell nucleus, paralleled by nuclear swelling	Export of early transcripts (mRNA) through nuclear pore central channels

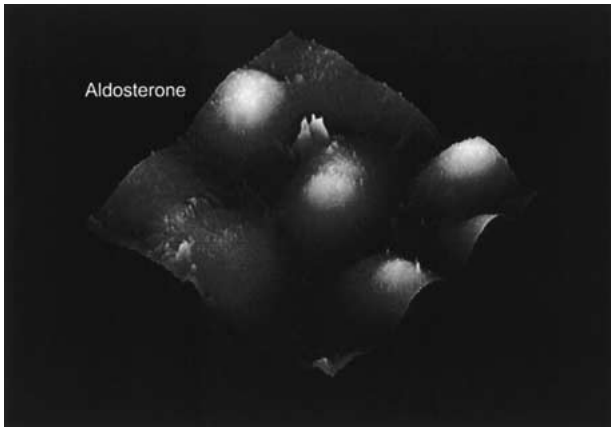

Fig. 6. AFM imaging of human umbilical vein endothelial cells (HUVEC). Shown is the apical endothelial surface. An individual HUVEC cell, exposed 3 days to aldosterone, has a volume of about 2000 femtoliter. Clearly visible are the swollen nuclei (light upper parts of cells). Cell heights are in the range of 5 μm (after Oberleithner et al., 2004).

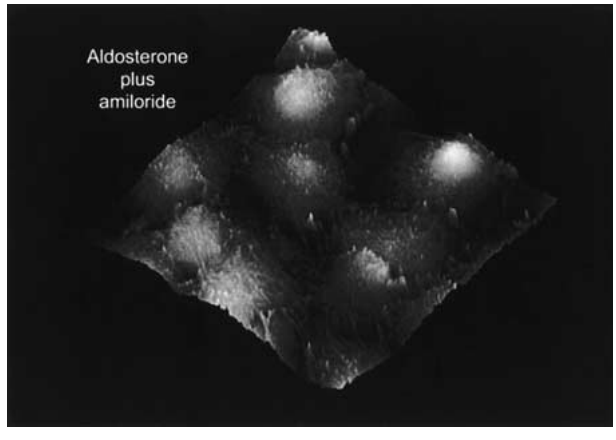

Fig. 7. AFM imaging of human umbilical vein endothelial cells (HUVEC). Shown is the apical endothelial surface. An individual HUVEC cell, exposed 3 days to aldosterone and then treated with a plasma membrane sodium channel blocker (amiloride) for one hour, has a volume of about 1400 femtoliter. Cells and nuclei appear shrunken as compared to cells in absence of the blocker (compare with Fig. 6). Cell heights are in the range of 3–4 μm (after Oberleithner et al., 2004).

steroid interaction with DNA response elements could explain changes in volume.

The changes of cell volume in response to aldosterone could be divided into two phases, a pregenomic and a genomic response (Oberleithner et al., 2003). The onset was immediate (within two minutes) and independent of the classical receptors since spironolactone was ineffective. This was typical for a pregenomic response. It smoothly intercalated with the genomic response, which was indicated by sensitivity to spironolactone 5 min after hormone exposure. Noteworthy was the transient nature of the volume change and the biphasic response observed in the nuclear compartment. The sharp volume increase of the nuclear compartment at the onset of the hormone response and the sharp volume decrease about 15 to 20 min later strongly indicated that the volume change occurred in the nucleus. It matched the previous observations of receptor import into the nucleus within two minutes and mRNA export into the opposite direction after 20 minutes. We proposed a model where the aldosterone-induced initial nuclear swelling was indicative for receptor import, while the late nuclear shrinkage indicated nuclear mRNA export (Oberleithner et al., 2003).

Amiloride

To our surprise, cariporide, a potent inhibitor of plasma membrane Na^+/H^+ exchange (Symons & Schaefer, 2001), did not block the volume changes. Usually, the antiporter mediates sodium uptake whenever a cell needs to gain volume (Hayashi, Szaszi & Grinstein, 2002). This situation should happen at the onset of aldosterone action when receptors, accompanied by water, move into the nucleus and thus shrink the cytosolic compartment (while the nucleus swells). In contrast to cariporide, amiloride was effective. A low dose of amiloride wiped out the aldosterone-induced volume increase, indicating that epithelial Na^+ channels mediated the volume changes across plasma membrane. Indeed, epithelial Na^+ channels have been shown to exist in vascular endothelial cells (Golestaneh et al., 2001). They are

regulated by aldosterone and require an intact cytoskeleton. Figures 6 and 7 show aldosterone-induced endothelial cell swelling and its inhibition by amiloride.

Conclusion and Outlook

The nuclear envelope is a smart barrier. Transport of molecules across this barrier is mediated by perfectly tuned nuclear pore complexes. Aldosterone and probably other steroid hormones regulate the nuclear pores and make them more or less permeable, depending on the metabolic state of the cell. From the physiological point of view this knowledge could be useful in understanding the signal cascade underlying steroid hormone action. From the medical point of view a clearly defined, hormonally induced transient permeability change of the nuclear barrier could help to specifically deliver material from the cytosol into the nucleus. Such treatments could be eventually applicable also in the human organism.

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