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Transient Permeability Leak of Nuclear Envelope Induced by Aldosterone

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Abstract. The mineralocorticoid hormone aldosterone controls fluid and electrolyte transport in target cells of the kidney and the cardiovascular system. Classic genomic aldosterone action involves the activation of cytosolic mineralocorticoid receptors and translocation into the cell nucleus where specific transcription processes are initiated. A key barrier of the intracellular signalling pathway is the nuclear envelope, which physically separates the nucleoplasm from the cytoplasm. It was shown recently that aldosterone changes ion conductivity of the nuclear envelope mediated by nuclear pore complexes. The latter are supramolecular nanomachines responsible for import and export of inorganic ions and macromolecules. The aim of the present study was to test whether aldosterone changes the macromolecule permeability of the nuclear envelope. Aldosterone-responsive *Xenopus laevis* oocytes were used as a model system. We isolated the cell nuclei at defined times after hormone injection. By means of confocal fluorescence microscopy and fluorescence-labelled dextrans we evaluated passive macromolecule import and export in isolated nuclei. 10 minutes after aldosterone injection nuclear envelope permeability of 10 kD dextran was found sharply increased. At the same time cell nuclei were found swollen by about 28%. Changes in nuclear volume and nuclear envelope permeability lasted 5 to 15 minutes and could be inhibited by the mineralocorticoid receptor blocker spironolactone. We conclude that aldosterone transiently changes the barrier function of the nuclear envelope. This short-lasting permeability change signals the start of a sustained transcription process that follows in response to steroids.

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Key words: Nuclear pore complex permeability — *Xenopus laevis* oocyte — Steroids — Cell nucleus — Transcription — mRNA export

Introduction

The nuclear envelope is a barrier that shields the chromatin from the rest of the cell and selectively controls the exchange of inorganic ions and macromolecules between nucleus and cytoplasm. In all eukaryotic cells the nuclear envelope is formed by two concentric, lipid bilayer membranes encircling the genetic material (Watson, 1955). This barrier is penetrated by nuclear pore complexes (NPCs), which serve as the crucial transport pathways for ions, metabolites and macromolecules. An individual NPC is a supramolecular structure formed by hundreds of proteins (so-called nucleoporins) and has an estimated molecular mass of ~125 MD (Reichelt et al., 1990). The most prominent feature of an NPC is the large central channel, ~10 nm in width and ~50 nm in length. In addition to the central channel pathway, structural and functional data indicate the existence of eight peripheral channels circularly arranged around the large central channel (Hinshaw, Carragher & Milligan, 1992). NPC can undergo dramatic conformational changes that strongly affect the electrical conductivity and macromolecule permeability of the central channel (Mazzanti, Bustamante & Oberleithner, 2001).

The nuclear envelope is involved in the action of different hormones. The first study, reported 39 years ago by Ito and Loewenstein (Loewenstein, 1965), showed changes in nuclear membrane permeability for inorganic ions in *Chironomus thummi* salivary gland cells after 20–OH ecdysone treatment. Recently we realized that *Xenopus laevis* oocytes respond to mineralocorticoids (Schafer et al., 2002). Aldosterone is known to bind to cytosolic mineralocorticoid receptors, which can be specifically inhibit-

ed by spironolactone, a competitive mineralocorticoid receptor antagonist. Hormone binding changes receptor conformation and translocates the receptor into the nucleus where it attaches to specific DNA regions. Transcription occurs and finally mRNA is exported through the nuclear pores in order to be translated into aldosterone-induced proteins. Some of these signalling steps can be followed in oocytes at the single-molecule level. By means of atomic force microscopy (AFM) we recently observed conformational changes of individual NPCs after aldosterone stimulation (Schafer et al., 2002).

The combination of nuclear envelope electrical conductance measurements (Danker et al., 1999) with AFM measurements of individual NPCs (Schafer et al., 2002; Shahin et al., 2001) led to the conclusion that transport of endogenous macromolecules (e.g., hormone receptors and mRNA) causes marked changes in NPC conformation, paralleled with electrical conductance changes of the nuclear envelope. Since electrical conductivity is related to inorganic ion movements (i.e., electrically charged atoms) rather than to movements of macromolecules (i.e., large molecules with comparatively few electrical charges) we performed in vitro studies on isolated nuclei of hormone-injected oocytes using fluorescent-labelled dextrans. These molecules are too large to penetrate the small NPC peripheral channels but freely diffuse through NPC central channels if open.

The present study describes a transient nuclear envelope leak for small macromolecules that occurs between 5 and 15 minutes after a single aldosterone injection.

Materials and Methods

Preparation of Oocyte Nuclei

Female *Xenopus laeves* were anaesthesized with 0.1% ethyl m-aminobenzoate methanesulfonate (Serva, Heidelberg, Germany) and parts of their ovaries were removed. The ovary clusters were stored in modified Ringer solution (in mM: 87 NaCl, 6.3 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10, HEPES, 100 U/100 μg penicillin/streptomycin, pH 7.4) at 4°C before use. The oocytes could be stored over 2–3 days in modified Ringer solution without changing their biological properties.

Before the experiments, stage-VI oocytes were dissected from the ovary cluster. Then, they were injected with H_2O containing 10^{-6} M aldosterone (stock solution: aldosterone dissolved in ethanol at a concentration of 10^{-3} M; Sigma, St. Louis, MO). For control experiments, H_2O containing 10^{-6} M aldosterone and 10^{-5} M spironolactone (stock solution: spironolactone dissolved in ethanol at a concentration of 10^{-3} M; Sigma) were coinjected. The volume of all injections was 50 nl per oocyte, which is less than 10% of total oocyte volume.

After defined time periods the injected oocytes were transferred from the modified Ringer solution into nuclear isolation medium (NIM) (in mM: 10 NaCl, 90 KCl, 2.0 $MgCl_2$, 10 HEPES, 1.1 EGTA and 1.5% polyvinylpyrrolidone (PVP, M_r = 40,000); titrated to pH 7.4), in which the cell nuclei were manually prepared and collected. The NIM was free of ATP and Ca^{2+} in order to stop the active nucleocytoplasmic transport immediately after nuclear isolation (Schafer et al., 2002). PVP was added to NIM because in presence of this osmotically active substance the nuclei maintained their original size (Danker et al., 2001). Thus, the nuclei were more resistant to mechanical stress during superfusion.

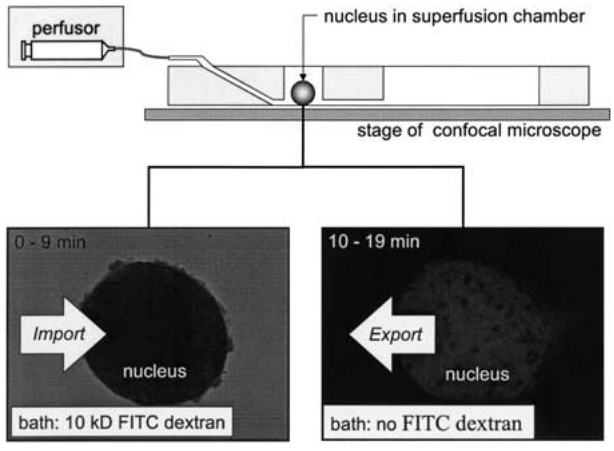

Fig. 1. Typical experimental set-up. Shown is the nuclear superfusion chamber with an oocyte nucleus attached to the bottom of the chamber. Images represent the time course of an experiment in which the nucleus was exposed to different bath solutions: 10 kD FITC dextrans dissolved in nuclear isolating medium (NIM) or NIM, free of any fluorescence-labelled dextrans. Arrows indicate the route of 10 kD FITC dextrans across the nuclear envelope.

Nuclear Fluorescence Measurements

After isolation the cell nucleus was picked up with a Pasteur pipette and transferred to a superfusion chamber filled with NIM. Care was taken to keep the nuclei in fluid at all times. The glass bottom of the fluid chamber was coated with Cell-tak® (Biosciences, Two Oak Park, Bedford, USA). Thus, the isolated cell nucleus, firmly attached to the glass surface, could be superfused with various solutions on the stage of a confocal microscope. A typical experimental setup is shown in Fig. 1. A perfusor, connected to the superfusion chamber, allowed complete exchange of the superfusate in about 20 seconds. In a typical experiment, 10 kD dextrans (concentration: 5 μM), labelled with FITC (fluoresceine isothiocyanate; Sigma) and dissolved in NIM, were superfused. Basically, we applied two solutions alternatively, one with and one without fluorescent dextrans. In order to quantify the fluorescence of 10 kD FITC-dextran outside and inside of the nucleus we used a confocal laser-scanning microscope (CLSM Fluoview, Olympus) with an argon/crypton ion laser. We utilized an excitation wavelength of 488 nm.

The underlying principle of exploring passive transport of macromolecules is that a change in the functional state of individual NPCs is accompanied by a change in the passive diffusion rate of fluorescence-labelled dextran. Comparison of diffusion rates under different experimental conditions allows conclusions on NPC macromolecule permeability (Enss et al., 2003). The diffusion of dextrans from cytoplasm to nucleus and vice versa inversely correlates with the molecular masses of the respective dextrans (nuclear exclusion limit between 17 and 41 kD), indicating that the nuclear envelope functions as a molecular sieve (Peters, 1984). Therefore, we used 10 kD dextran to test for the NPC diffusional pathway.

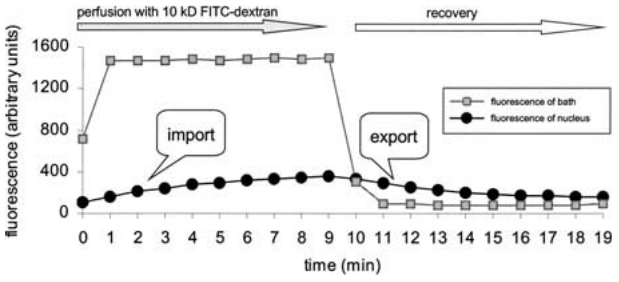

Fig. 2. Representative experiment of an individual nucleus. The fluorescence of the nucleus and of the bath solution is shown as a function of the superfusion time.

A representative experiment is shown in Fig. 2. The measurement (15 s per image) started with the superfusion of 10 kD FITC dextran. This part of the superfusion phase served to test for the passive diffusional macromolecule pathway directed from cytoplasm to nucleus (macromolecule nuclear import). Then, between the 9^{th} and 10^{th} minute of superfusion (i.e., when nuclear fluorescence was saturating), a superfusion solution free of 10 kD FITC dextran was offered. This part of the superfusion phase served to test for the passive diffusional macromolecule pathway directed from nucleus to cytoplasm (macromolecule nuclear export).

In order to evaluate the fluorescence quantitatively, we determined nuclear (inside) and bath (outside) fluorescence intensity of each scanned image (Fig. 2). Each nuclear fluorescence intensity value (in arbitrary units) was related to the respective bath fluorescence intensity value measured at identical time and a ratio was formed (inside fluorescence/outside fluorescence). The initial slope was derived from the straight line set through the first three ratio values. It directly correlates with macromolecule nuclear envelope permeability.

Nuclear Diameter Determination

For nuclear diameter determination the respective image of a fluorescent nucleus was imported into MetaMorph® (Universal Imaging Corporation, Downingtown, PA). This software allows measurement of the area (in number of pixels) of the nucleus of individual nuclear images by using a manually driven digital curser. The nuclear diameter was calculated by using the area (i.e., the cross-sectional area of a sphere) and the mathematical formula for diameter (d):

$$d(\text{pixel}) = 2 \times \sqrt{\text{area}/\pi}$$

A pixel of the scanned image corresponded to 2.5 μm of the real nucleus. So the diameter (μm) was calculated in the following way:

$$d(\mu\text{m}) = 5 \times \sqrt{\text{area}/\pi}$$

Statistics

Data of experiments are presented as mean values ± standard error of the mean (SEM). Each experimental group was statistically compared with its respective control. Statistical analysis was performed by unpaired Student's t-test. Significant difference was accepted at $P \leq 0.05$. Numbers in parenthesis (n) present numbers of nuclei tested for each group.

Results

After aldosterone injection the oocyte nucleus changed its volume. Figure 3 shows changes of the nuclear diameter in response to the hormone, measured over a time scale of 20 minutes. The initial control value of the nuclear diameter was 471 ± 9.0 μm. Ten minutes after aldosterone injection we observed a sharp increase of the diameter to 511 ± 9.8 μm. This indicated an increase of nuclear volume by about 28%. Five minutes later, nuclear volume approached the initial control value. In order to test for specificity of the aldosterone response we coinjected the receptor antagonist spironolactone together with aldosterone. Coinjection completely blocked aldosterone-induced nuclear swelling.

Then, we investigated the influence of aldosterone injection upon 10 kD FITC dextran permeability of the nuclear envelope. We superfused the same batch of nuclei that had been used for the diameter measurements. Unfortunately, quite a number of nuclei got damaged during superfusion, so the number of experiments in the "permeability study" was less than that of the "diameter study". We measured dextran permeability (i.e., the fluorescence ratio of nuclear fluorescence over outside fluorescence) in nuclei in response to aldosterone at a time scale of 20 minutes in absence and presence of the aldosterone antagonist spironolactone. We measured both the kinetics of passive dextran perfusion into the nucleus (import pathway, Fig. 4) and out of the nucleus (export pathway, Fig. 5). As mentioned above, we observed a rather dramatic increase in nuclear size 10 minutes after aldosterone injection. However, a comparison of the initial slopes derived from the fluorescence ratio measurements in nuclei of different sizes is only meaningful when surface/volume ratios are taken into consideration. Therefore, we calculated initial slopes by considering the respective surface volume ratios. We assumed that nuclear envelope surface remained constant over the course (20 minutes) of the experiments. In figures 4 and 5 results are shown after appropriate corrections for the observed changes in surface/volume ratios.

Initial nuclear import and export slopes for dextran were found significantly increased 10 minutes after injection of aldosterone (fluorescence ratio for import rate: 0.051 ± 0.0037 and for export rate: 0.037 ± 0.0023). Already 5 minutes later, slopes were found again at initial control values. In contrast, we could not observe any significant change in the initial slopes when the receptor antagonist spironolactone was coinjected with aldosterone. The results of Figs. 4 and 5 allow at least three statements: (i) aldosterone affects the nuclear envelope permeability transiently, (ii) the permeability change is mineralocorticoid specific and finally, (iii) the passive permeability leak is symmetric. The last conclusion was

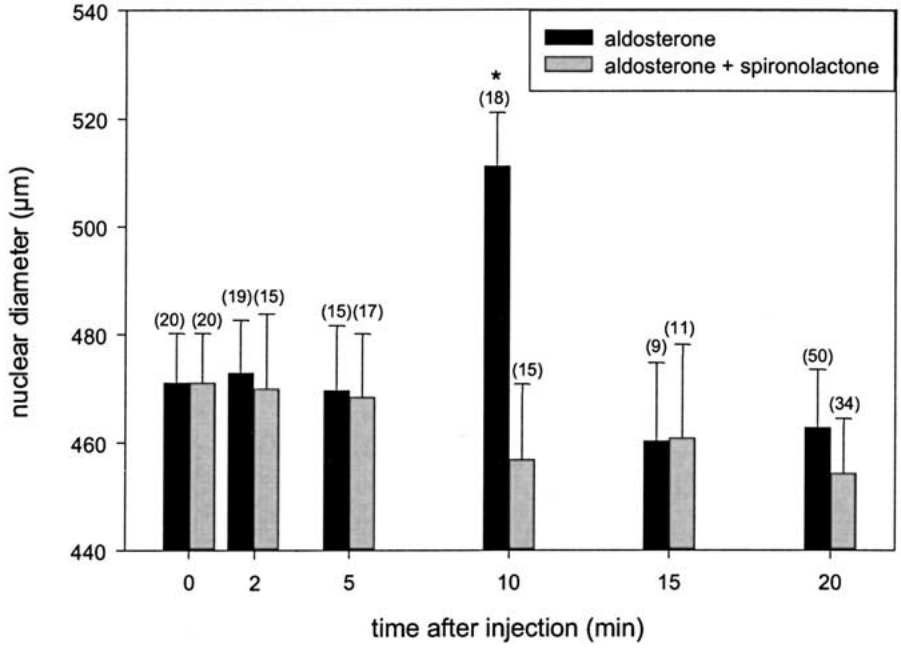

Fig. 3. Time course of aldosterone action on nuclear diameter of oocyte nuclei. Numbers in parenthesis present numbers of nuclei tested for each group. Asterisk indicates a significant difference between the mean data of the aldosterone experiments and those of the aldosterone + spironolactone experiments.

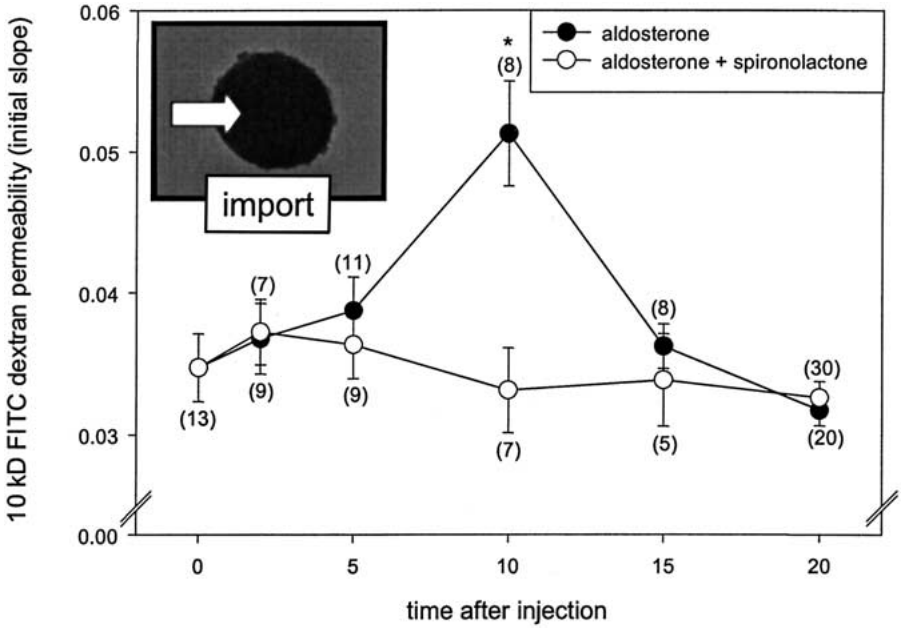

Fig. 4. 10 FITC dextran permeability ("dextran import", given as initial slope; for details *see* Methods) shown as a function of time after either aldosterone or aldosterone + spironolactone injection. 10 minutes after aldosterone injection, there is a significant permeability leak of the nuclear envelope, which is inhibited by spironolactone. Asterisk indicates significant difference between the two 10-minute values.

derived from the observation that inward- and outward-directed dextran fluxes were similar (*compare* data of Figs. 4 and 5).

Discussion

The mature *Xenopus laevis* oocyte is a suitable model cell for studying the barrier functions of the nuclear envelope. We used stage-VI oocytes (Dumont, 1972), since they are usually inactive in terms of transcription, but can be activated by exogenous stimuli (Golden, Schafer & Rosbash, 1980). They respond to aldosterone with a genomic signalling cascade that involves the cell nucleus. Beside the classic genomic pathway, aldosterone elicits fast "pre-genomic" effects at the level of plasma membrane ion transporters or cytosolic enzymes (Oberleithner et al., 1987; Harvey & Higgins, 2000). First postulated as "ion hypothesis of gene activation" more than 40 years ago by Kroeger (1966) and later confirmed in our laboratory (Wunsch et al., 1993), a change of intracellular ion composition, in particular a change

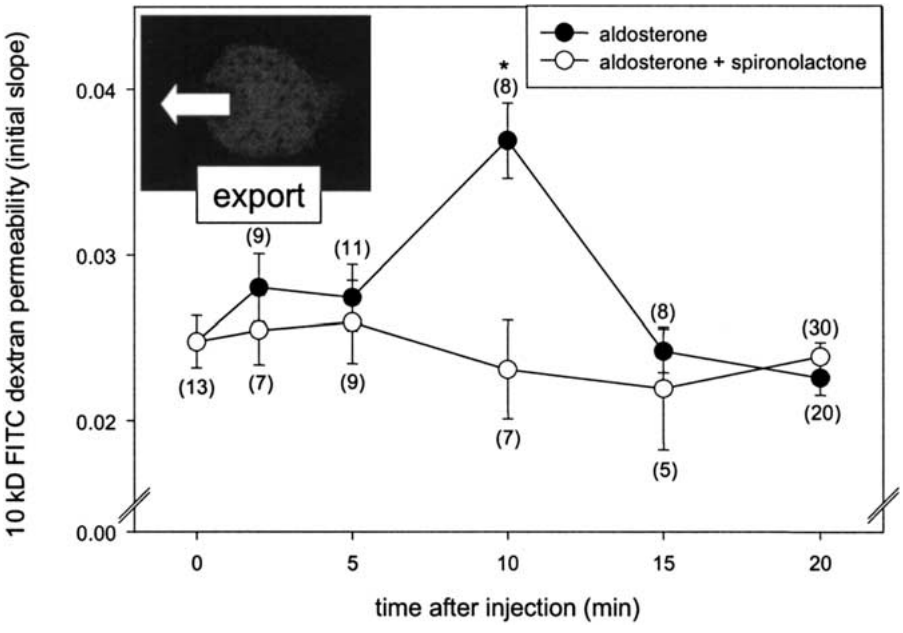

Fig. 5. 10 FITC dextran permeability ("dextran export", given as initial slope; for details *see* Methods) shown as a function of time after either aldosterone or aldosterone + spironolactone injection. 10 minutes after aldosterone injection there is a significant permeability leak of the nuclear envelope, which is inhibited by spironolactone. Asterisk indicates significant difference between the two 10 minute values.

in free Ca^{2+} and H^+, seems necessary for steroid-induced gene activation. The genomic response to steroid hormones involves the binding of the steroid to cytoplasmic receptors, which then migrate into the nucleus. In this scenario, the NPCs of the nuclear envelope are the only possible pathways that functionally connect nucleoplasm with cytoplasm.

The NPC is a supramolecular nanomachine that can dilate during translocation in order to transport cargo with molecular masses in the millions of Daltons. Such large particles cannot diffuse through the NPC central channel unless conformational changes occur (Daneholt, 1997) and alter NPC permeability (Paine Mome & Horowitz, 1975). Thus, nuclear pore-complex permeability cannot be adequately described by a certain "fixed" value but, in contrast, seems highly variable. Passive diffusion of 10 kD dextrans through the central channel can be decreased by antibodies against the glycoproteins gp62 and gp210, nucleoporins that physically and functionally define the cytoplasmic entrance of the NPC central channel (Greber & Gerace, 1992; Enss et al., 2003). Furthermore, the filling state of the perinuclear Ca^{2+} stores controls NPC conformation inasmuch Ca^{2+} store depletion blocks the passive diffusion of intermediate-sized molecules (Stehno-Bittel, Perez-Terzic & Clapham, 1995; Wang & Clapham, 1999). Finally, cytosolic ATP and Ca^{2+} are supposed to be key elements that regulate nuclear envelope ion conductivity by structural NPC reorganization (Mazzanti et al., 1994; Shahin et al., 2001).

In the present study we asked the question whether the nuclear envelope changes its permeability for macromolecules in response to steroids. Indeed, 10 min after aldosterone addition nuclear envelope dextran permeability was found significantly increased. This macromolecule permeability change was transient. It paralleled the electrical ion conductivity change observed previously (Schafer et al., 2002). The latter could be inhibited by the transcription blocker actinomycin D (Oberleithner et al., 2003) or the chelation of intracellular free Ca^{2+} (Schafer et al., 2003).

The nature of the nuclear envelope leak is still unknown. It could be closely related to the phenomenon that nuclei transiently swell in response to aldosterone. Transient hormone-induced nuclear swelling has been recently observed also in aldosterone-responsive endothelial cells (Oberleithner et al., 2003). It was explained by the movement of activated hormone receptors into the cell nucleus and decondensation processes of the chromatin in the course of gene transcripton. We know that the nuclear envelope under physiological conditions is usually folded by about 2.6 times according to recent literature (Danker et al., 2001). Therefore, nuclear swelling can occur without any change in true envelope surface. Thus, nuclear swelling leads to the unfolding of the nuclear envelope. This could more "efficiently" expose NPCs to the cytosol. As a consequence, both electrical conductivity and macromolecule permeability could be increased. Another, yet more remote, explanation could stem from previous observations that aldosterone can increase the absolute number of NPCs per nucleus (Oberleithner et al., 1994). Such findings were made in response to aldosterone in kidney cells 6 hours after hormone exposure. However, it is rather unlikely that within 10 minutes a similar phenomenon could have occurred.

Finally, the transient aldosterone-induced leak of the nuclear envelope could be due to conformational changes of individual NPCs. According to the liter-

ature, a single NPC is equipped with 8 peripheral channels and one central channel (Hinshaw et al., 1992; Pante & Aebi, 1993; Shahin et al., 2001). The peripheral channels are small and located in the NPC ring structure. The central channel is wide and located in the NPC centre. The peripheral channels could function like ion channels with small electrical conductivity and specific gating characteristics (Mazzanti et al., 2001). The central channel could function like a nanomachine that transports macromolecules of different sizes and thus undergoes dramatic changes in conformation (Perez-Terzic et al., 1996; Shahin et al., 2001; Jaggi et al., 2003). The central channel is usually plugged with cargo. When plugged, electrical NPC conductivity is expected to be low unless the small peripheral channels are electrically open (Mazzanti et al., 2001). In the present study, the 10 kD dextran most likely moves through the NPC central channel. This conclusion is simply derived from the fact that 10 kD dextran is too large to sneak through any of the 8 small peripheral NPC channels. However, as mentioned above, NPC central channels are usually plugged and thus cannot serve as functional pathways for the dextrans. This apparent discrepancy can be explained as follows:

The electrical conductivity is based on NPC peripheral Channels (Mazzanti et al., 2001). When a cell is activated by steroids, transport cargo (e.g., activated receptors, transcription factors) interacts with individual NPCs. Since this physical interaction occurs on the NPC surface, at the locations of the peripheral channels, there is a transient electrical conductivity change detectable (Schafer et al., 2002). However, no electrical conductivity change occurs when the central channel is occupied by antibodies (Danker et al., 1999). The later study strongly supports the hypothesis that usually the central channel is plugged by dielectric transport cargo and thus antibody binding to the cytoplasmic central channel mouth will not affect ion conductivity (i.e., a plugged central channel cannot conduct ions and thus any "antibody-central channel" interaction is ineffective on NPC electrical conductivity). 10 minutes after aldosterone exposure there is a sharp increase in both electrical conductivity (Schafer et al., 2002) and dextran permeability (this study). At the level of individual NPCs a likely explanation is that a distinct population of NPCs of a single cell nucleus becomes transiently leaky. This leakage pathway must involve the central channel due to the observed increased dextran permeability. Possibly, for a short period of time, for about 10 minutes, a small "aldosterone-sensitive population of NPCs" has open (unplugged) central channels. Both inorganic ions and macromolecules can readily travel through the central channel pathway. We can only speculate on the physiological relevance of this phenomenon: Steroid-induced intracellular signalling involves only a minor population of NPCs. It is likely that the transient macromolecule permeability leak is mediated by only a small percentage of "aldosterone-sensitive" NPCs that allow rigorous local exchange of material. It is tempting to speculate that specialized NPCs located close to me nucleoplasmic sites of hormone-specific gene transcription are responsible for the physiological leak of the nuclear envelope. The short-lasting leak initiates transcription. This hypothesis, however, needs to be further tested.

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