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Intracellular Calcium: A Prerequisite for Aldosterone Action

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Abstract. Transport of salt and water in various tissues is under control of the mineralocorticoid hormone aldosterone. As a liphophilic hormone, aldosterone diffuses through the plasma membrane and, then, binds to cytosolic mineralocorticoid receptors in the target cells. After binding to nuclear pore complexes, the activated receptor is translocated to the nucleus where transcription processes are initiated. After a lag period of about 20 minutes hormone-specific early mRNA transcripts leave the nucleus through nuclear pores. Some of the steps in this cascade can be followed by electrophysiology in *Xenopus laevis* oocyte nuclei. In addition to the genomic pathway, aldosterone exerts a rapid pre-genomic response that involves an increase in intracellular calcium. In this study, we tested for the potential role of Ca^{2+} in the genomic response of the hormone. We measured the electrical resistance across the nuclear envelope in response to aldosterone, in presence and absence of intracellular Ca^{2+}. Nuclear envelope electrical resistance reflects receptor binding to the nuclear pore complexes (''early'' resistance peak, 2 minutes after aldosterone), ongoing transcription (''transient'' resistance drop, 5–15 minutes after aldosterone) and mRNA export (''late'' resistance peak, 20 minutes after aldosterone). Preinjection of the Ca^{2+} chelator EGTA eliminated all electrical responses evoked by aldosterone. The transient resistance drop and the late resistance peak, induced by the hormone, were prevented by the transcription inhibitor actinomycin D, coinjected with aldosterone, while the early resistance peak remained unaffected. We conclude that (i) the presence of intracellular Ca^{2+} is a prerequisite for the genomic action of aldosterone. (ii) Intracellular calcium plays a role early in the signaling cascade, either in agonist-receptor interaction, or receptor transport/docking to the nuclear pore complexes.

Key words: *Xenopus laevis* oocyte — Transcription — Mineralocorticoid receptor — Actinomycin D

Introduction

In target cells, aldosterone can act fast (seconds to minutes) followed by a sustained response that involves the cell nucleus. The fast pre-genomic effects are characterized by their insensitivity to receptor antagonists (e.g., spironolactone), to inhibitors of transcription (e.g., actinomycin D) and to blockers of translation (e.g., cyclohexamid). Pre-genomic action usually involves second messengers, such as inorganic ions, cyclic AMP or various protein kinases. Calcium is one of the second messengers and its increase is known to be among the first intracellular signals in response to aldosterone. This has been demonstrated in human mononuclear leukocytes (Wehling et al., 1990), renal epithelial cells (Gekle et al., 1996) vascular smooth muscle cells (Wehling et al., 1994) porcine endothelial cells (Schneider et al., 1997a) and rat distal colon (Doolan & Harvey, 1996). Following aldosterone application in endothelial cells, Ca^{2+} increases immediately and reaches a plateau within 2–3 min (Wehling et al., 1994) followed by transient cell swelling (Schneider et al., 1997b). As shown in distal colon, this increase of intracellular calcium is assigned to the fast pre-genomic effects of aldosterone mediated by the activation of protein kinase C (Doolan & Harvey, 1996; Doolan, O'Sullivan & Harvey, 1998). There is strong evidence that the pre-genomic hormone response can influence the genomic processes. It is the current view that clearly separated pre-genomic and genomic pathways are unlikely to occur. Rather, a pre-genomic change in intracellular inorganic ions or organic substances, evoked by aldosterone in seconds, influences the

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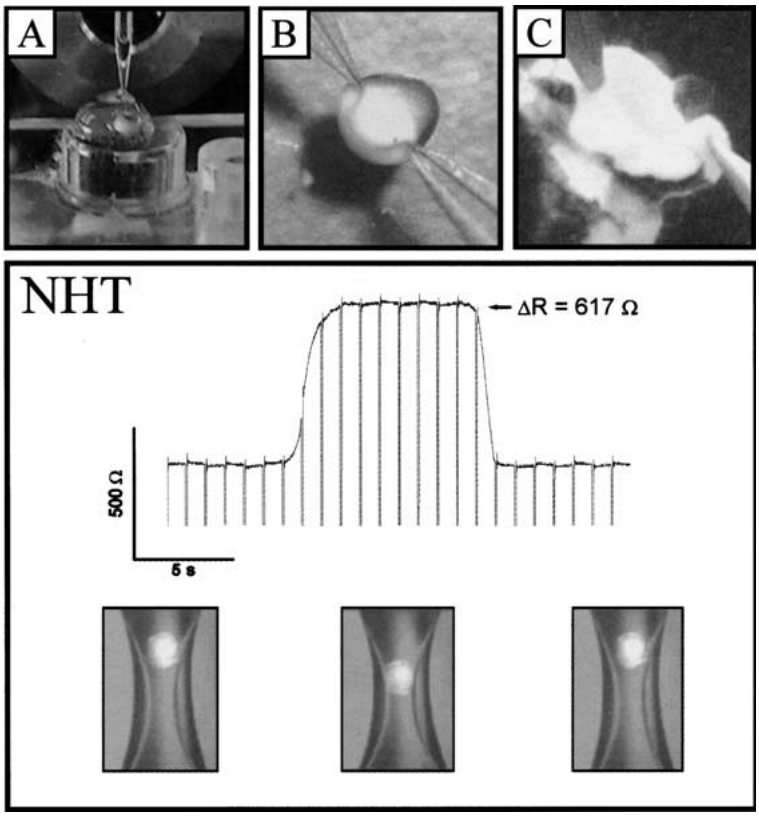

Fig. 1. Preparation of nuclei and electrical measurements. (*A*) Hormone microinjection into the oocyte. (*B*, *C*) Isolation of a *Xenopus laevis* stage-VI-oocyte nucleus. *Lower part:* Nuclear envelope electrical resistance (NEER) measurements with the nuclear hourglass technique (NHT). Electrical tracing (*center*) shows a representative NEER measurement in a cell nucleus of an aldosterone-injected oocyte. The three images at the bottom part of the figure show the tapered glass of the NHT with a nucleus inside the glass tube. The position of the nucleus corresponds to the electrical changes in the tracing above.

genomic action through intracellular crosstalk mechanisms. This view is supported by observations that second messengers can modulate genomic mineralocorticoid and glucocorticoid actions (Lim-Tio, Keightley & Fuller, 1997; Nordeen, Moyer & Bone, 1994).

By using an electrophysiological approach, the nuclear hourglass technique (NHT), we can trace the intracellular aldosterone response at the level of the nuclear envelope (Schafer et al., 2002). This barrier, located between cytosol and nucleoplasm, is penetrated by nuclear pore complexes, which serve as the crucial transport pathways for the import of hormone receptors and the export of mRNA. Whenever receptors bind to the pore complexes or mRNA exits through their central channels, an electrical resistance peak can be measured. Moreover, an electrical resistance drop occurs while the cell undergoes transcriptional processes. We used this characteristic electrical response (early peak, transient drop and late peak) to investigate the role of Ca^{2+} in aldosterone-induced intracellular signaling. We performed the experiments in nuclei of *Xenopus laevis* oocytes since they electrically respond to aldosterone and, due to their large size, can be handled more easily compared to cultured mammalian cells. We found that intracellular Ca^{2+} chelation eliminates the aldosterone-induced signal cascade and thus prevents the classical genomic response of the hormone.

Materials and Methods

Preparation of Oocyte Nuclei

Female *Xenopus laevis* were anesthetized with 0.1% ethyl m-aminobenzoate methanesulfonate (Serva, Heidelberg, Germany) and their ovaries removed. Stage-VI oocytes were dissected from ovary clusters and stored in HEPES-buffered solution (in mM: 87 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 100 U/100 μg penicilline/streptomycine, pH 7.4) before use.

For the calcium-depletion experiments, 25 nl of 400 mM EGTA solution were first microinjected into oocytes (Fig. 1) and, after a 15-min incubation period in calcium-free solution (in mM: 87 NaCl, 3 KCl, 1 $MgCl_2$, 10 HEPES, 100 U/100 μg penicilline/streptomycine, pH 7.4), injected a second time either with 50 nl of aldosterone (10^{-6} M) or of solvent (ethanol). Since the single-oocyte volume is approximately 1000 nl, intracellular EGTA and aldosterone concentrations of 10 mM and 2×10^{-8} M, respectively, were achieved. To block aldosterone-induced transcription, actinomycin D (10^{-5} M) was coinjected with aldosterone. Then, nuclei were isolated between 2 to 30 min after the second injection (Fig. 1).

For isolation of the nuclei, oocytes were transferred into nuclear isolation medium composed of (in mM): 10 NaCl, 90 KCl, 2.0 $MgCl_2$, 1.1 EGTA, 10 HEPES, titrated to pH 7.4. Furthermore, we added 1.5% polyvinylpyrrolidone (Mr = 40,000; Sigma) to compensate for the lack of macromolecules in the isolation medium mimicking the intact cytosol. The presence of polyvinylpyrrolidone

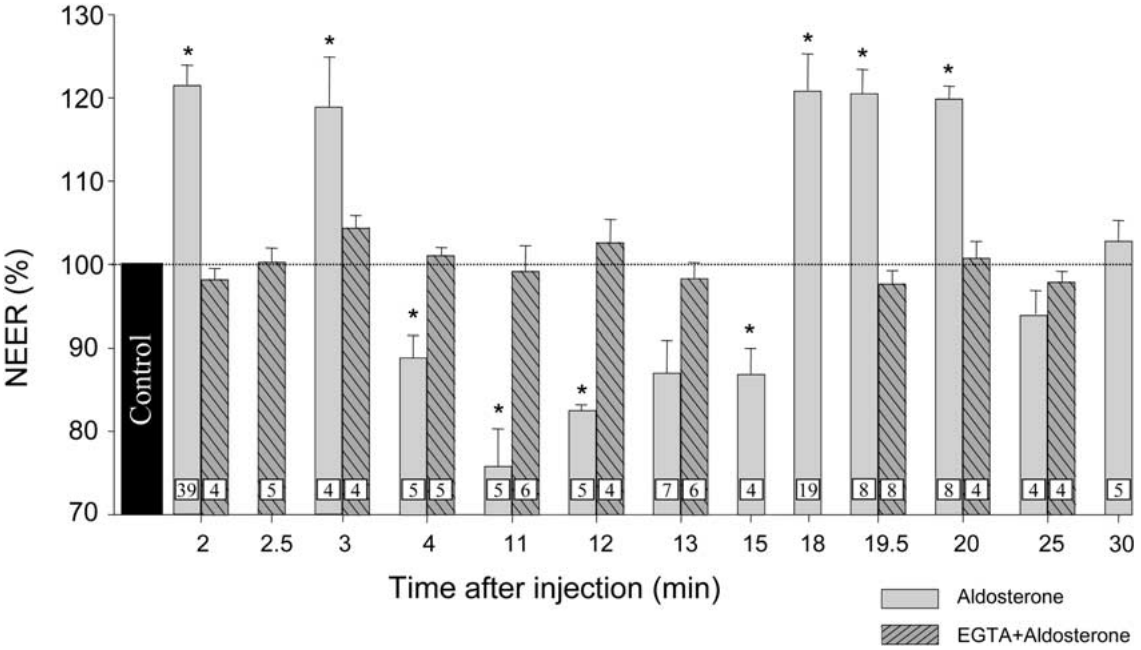

Fig. 2. Nuclear envelope electrical resistance (NEER) related to corresponding control values is shown as a function of time after either aldosterone injection or EGTA pre-injection plus subsequent aldosterone injection. Number of nuclei is given in the columns. Asterisks indicate mean NEER values significantly different from the control value.

is of crucial importance to prevent nuclear swelling, which instantaneously occurs during isolation in pure electrolyte solution (Danker et al., 2001).

Nuclear Hourglass Technique (NHT)

The technical aspects of the method and its application in isolated cell nuclei have been described in detail (Danker et al., 1999, 2001). In short, the method is based on a tapered glass tube, which narrows in its middle part to two-thirds of the diameter of the nucleus (Fig. 1). A current of 1 mA is injected via two Ag/AgCl electrodes through either end of the glass tube. The voltage drop across the cell nucleus is measured with two conventional microelectrodes. These microelectrodes are positioned near the tapered part of the capillary opposite to the current electrodes to measure the electrical resistance of the fluid column between them.

When the cell nucleus is gently sucked into the tapered part of the glass capillary by gentle fluid movement the whole current flows now through the accessible parts of the nuclear envelope (NE). The resulting rise in total electrical resistance indicates the NE electrical resistance (NEER). Since current and voltage are simultaneously measured, the resistance can be calculated online and monitored during the measurements (Fig. 1).

In the aldosterone, EGTA/aldosterone and actinomycin D/aldosterone experiments, nuclei isolated after different time lags (2–30 min) after hormone injection were brought into the NHT capillary filled with HEPES-buffered solution. Twenty seconds later, NEER measurements were started.

Statistics

Results are expressed as mean values ± SEM in % of the corresponding control values, n indicates the number of experiments. Statistical analysis was performed by Student's t-test. Significant difference was accepted at $p < 0.05$. Each experimental group was compared with its own control and prepared and analyzed simultaneously.

Results

Figure 2 shows NEER measurements over a time scale of 30 minutes. Two minutes after aldosterone injection NEER increases sharply. This early NEER peak disappears a few minutes later. A transient NEER decrease below the initial electrical resistance value follows. It takes about 10 minutes. Then, NEER rises again above the initial control values. This late NEER peak is again transient. NEER returns to the initial control value about 25 minutes after aldosterone injection. This is a characteristic electrical response to aldosterone, as previously reported (Schafer et al., 2002). When EGTA is pre-injected, the electrical response is completely eliminated. Although we did not observe changes in the absolute initial NEER values, the response to aldosterone is missing under these conditions. Furthermore, we tested whether the transcription inhibitor actinomycin D was effective. Although we knew from previous experiments that the late NEER peak was sensitive to actinomycin D, we had not yet tested whether the early peak and the transient drop could be inhibited by the transcription blocker. Figure 3 summarizes the results. Not surprisingly, the early NEER peak was insensitive to actinomycin D, indicating that this change in NE resistance was not due to any transcription processes. However, the

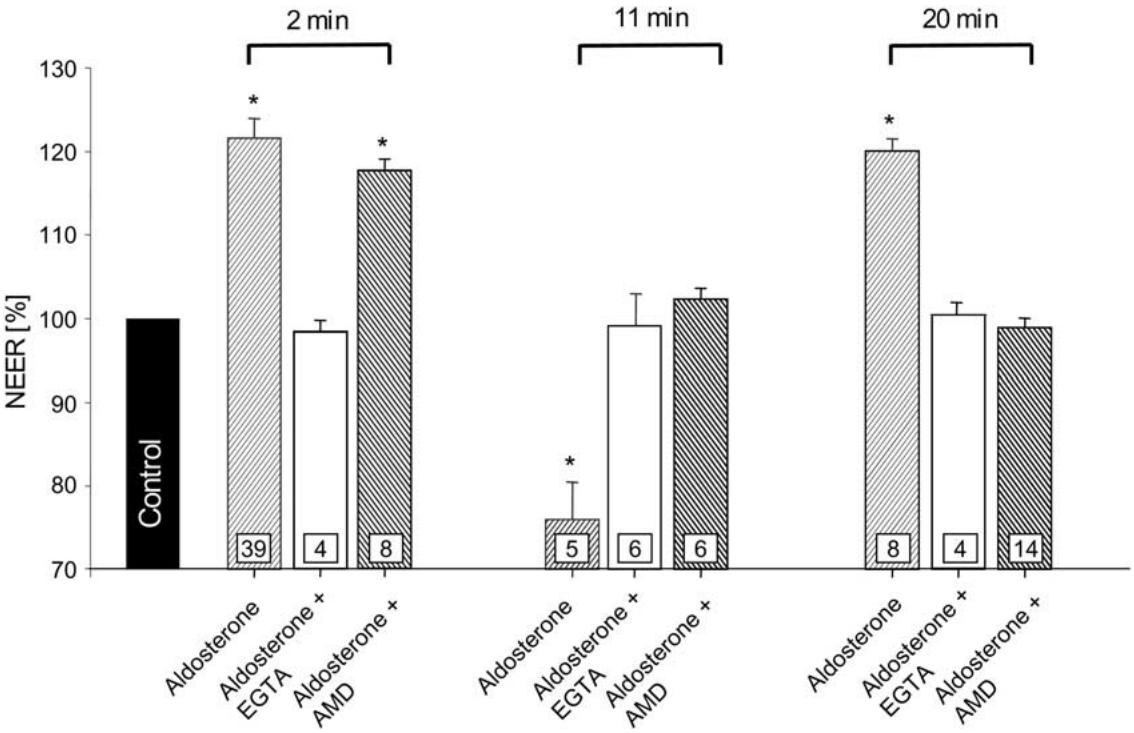

Fig. 3. NEER related to the corresponding control values (*control* = solvent injection) is shown after injection with aldosterone, with EGTA pre-injection plus subsequent aldosterone injection and aldosterone and actinomycin D coinjection. Experiments were performed 2, 11 and 20 minutes after the injections. The asterisk indicates a mean NEER value significantly different from the control value.

transient NEER drop was eliminated by the transcription blocker. From the data of Fig. 3 it is concluded that Ca^{2+} chelation interacts early with the hormone cascade, i.e., before transcription processes occur.

Discussion

Aldosterone is the major steroid hormone that regulates electrolyte transport across cell membranes in different target tissues. The intracellular hormone mechanism involves the activation of a genomic pathway that neccessarily includes transport processes across the nuclear envelope. With regard to the diversity of steroid hormone actions, observed over the past years, it has become clear that the classic genomic action does not explain all aspects of steroid hormone signaling.

Besides the genomic effect described above there is a fast pre-genomic response. Obviously, aldosterone may act also by binding to a still putative membrane receptor unrelated to the superfamily of intracellular steroid receptors (Haseroth et al., 1999). A typical example for a receptor/effector-cascade in pre-genomic steroid signaling involving non-classic membrane receptors are rapid aldosterone effects on the second messenger system. A two-step model has been proposed, which combines the fast pre-genomic effects and the delayed genomic response of target cells upon aldosterone application (Wehling, 1995). This model emphasizes that early alterations in intracellular signaling and membrane ion transport, caused by pre-genomic effects of aldosterone, controls classic genomic action through intracellular molecular crosstalk. Calcium is a crucial second messenger in the context of the rapid aldosterone response (Gekle et al., 1996; Harvey & Higgins, 2000), even in mineralocorticoid-receptor knockout mice (Losel & Wehling, 2003). The source of the cytosolic Ca^{2+} increase triggered by aldosterone can vary depending on the different cell types. Ca^{2+} can be released either from intracellular stores or can flow into the cell from the extracellular space across plasma membrane (Wehling, 1995). The present study was performed to elucidate the role of intracellular calcium in mediating the crosstalk between the pre-genomic and genomic route of aldosterone action.

The nuclear envelope is a crucial barrier between cytosol and nucleus that can be functionally characterized by electrical methods (Mazzanti, Bustamante & Oberleithner, 2001). The NE is penetrated by nuclear pore complexes, which are the only mediators for macromolecule transport between cytoplasm and nucleus. The nuclear pore complexes serve as selective barriers in the control of gene activation (Gorlich & Mattaj, 1996; Nigg, 1997).

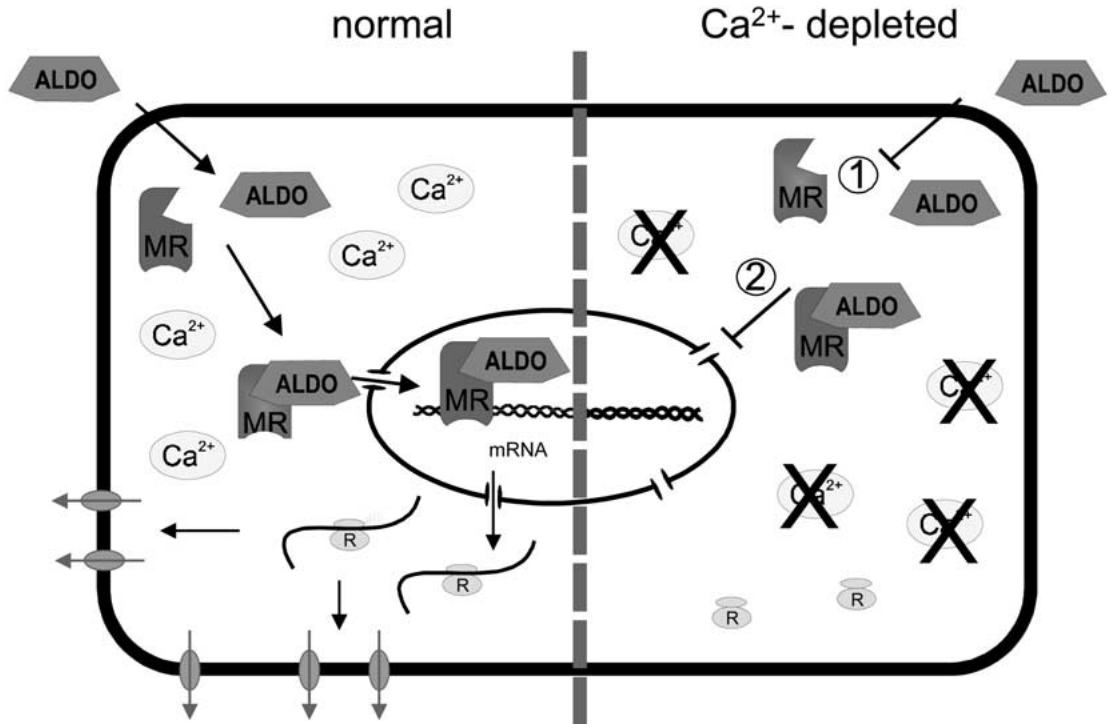

Fig. 4. Aldosterone-induced signaling under physiological conditions (normal intracellular Ca^{2+} response to aldosterone) and under Ca^{2+}-depleted conditions (Ca^{2+} chelation by EGTA). Under normal conditions (i.e., when cytosolic Ca^{2+} is available) aldosterone is known to bind to mineralocorticoid receptors located in the cytosol. Hormone-binding changes protein conformation and translocates the receptor into the nucleus where it attaches to specific DNA. Transcription occurs and finally mRNA is exported through the nuclear pores in order to be translated into aldosterone-induced proteins. When intracellular Ca^{2+} is chelated, a variety of cellular mechanisms, such as the adequate response to aldosterone, are probably disrupted. The location of the block is still unknown. It could be at the level of agonist-receptor interaction (number *1* in the figure) or at the level of receptor transport/docking to the nuclear pore complexes (number *2* in the figure). As a result, receptors would not be translocated to the nucleus. Therefore, the genomic response to aldosterone is missing.

Macromolecular translocation along nuclear pores results in electrical signals that can be detected by patch-clamp techniques (Bustamante & Varanda, 1998). We used the nuclear envelope electrical resistance as an indicator for aldosterone-induced cytosolic signaling into the cell nucleus. In a previous study we realized that, most likely, receptor docking to the cytosolic side of the nuclear pores and mRNA export from the nucleus into the cytosol through the central channels of the nuclear pores exert typical changes in electrical resistance (Schafer et al., 2002). Here, we term aldosterone effects "pre-genomic" when they involve mineralocorticoid receptor activation and docking to the nuclear pores and "genomic" when they originate from the inside of the cell nucleus. Since the application of the NHT allows such a crude functional separation, we chose this method to test for Ca^{2+} sensitivity.

With the injection of EGTA (estimated final oocyte concentration after EGTA injection is about 10 mM) we clamped intracellular Ca^{2+} to presumably very low levels independently of aldosterone.

The fact that the aldosterone response was completely eliminated by intracellular Ca^{2+} chelation indicates that intracellular Ca^{2+} is of crucial importance for the late genomic response. Surprisingly, already the early electrical peak, 2 minutes after aldosterone injection, was eliminated. This indicates that Ca^{2+} plays a decisive role already at the very beginning of the signal cascade (Fig. 4). Whether Ca^{2+} is required for hormone-receptor interaction, for vectorial transport of the activated receptor to the nuclear pores or for the docking process itself is yet unknown. Further experiments titrating cytosolic Ca^{2+} to specific values (e.g., values between 50 and 500 nM) over defined time periods (e.g., periods between seconds and minutes) should more specifically focus on the potential relevance of the aldosterone-induced Ca^{2+} signal.

The actinomycin D experiments in the present study confirm that the late electrical peak reflects part of the genomic route, as shown previously (Schafer et al., 2002). They also show that the transient decrease of the NE electrical resistance (transient resistance drop 5–15 minutes after aldosterone injection) can be ascribed to transcription, thus being part of the genomic response. The lack of effect of actinomycin D upon the early peak clearly indicates

that the early electrical signal at the level of the NE is pre-genomic.

As mentioned earlier, a rise in intracellular calcium concentration is the typical rapid response of a target cell when exposed to aldosterone (Gekle et al., 1996; Schneider et al., 1997; Harvey & Higgins, 2000; Golestaneh et al., 2001). It occurs a few seconds after hormone application and is usually transient in nature. It is, beyond doubt, a pre-genomic cellular event and possibly involves mineralocorticoid receptor dynamics. Mineralocorticoid receptors are known to be associated with cytoskeletal structures (Jalaguier et al., 1996). Aldosterone abolishes such interactions and, obviously, allows mineralocorticoid receptors to move on. This could be a crucial step of receptor translocation into the cell nucleus and calcium ions could play a role in this scenario.

In conclusion, the intracellular calcium increase elicited by aldosterone in target cells is most likely a prerequisite for hormone action. Only in the presence of appropriate concentrations of ionized calcium, a cell can properly respond to the steroid.

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