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Rapid Nongenomic Effects of Aldosterone in Mineralocorticoid-Receptor-Knockout Mice

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In addition to genomic effects of aldosterone, rapid nongenomic effects of steroids have been reported in various tissues that were clearly incompatible with a genomic action of aldosterone. Rapid effects of aldosterone involve second messengers such as calcium and cAMP. Specific high affinity binding sites for aldosterone have been characterized in membranes for different cells, which probably transmit those rapid steroid effects. To date, it is unclear if these binding sites are modified classical mineralocorticoid receptors (MR) or if they represent an unrelated receptor protein. The aim of the present study was to investigate whether rapid aldosterone action still occurs in the absence of the classical MR. For this purpose we used the model of MR knockout mice. Rapid effects were analyzed in skin cells, measuring intracellular calcium and cAMP levels after stimulation with aldosterone. We found that rapid effects are not only present in MR knockout mice, but that the effects are even larger than in wild-type mice cells. The results of the present study demonstrate that the classic MR is dispensable for rapid aldosterone action. The study, thus, proves that a receptor different from the classic intracellular receptor is involved in rapid aldosterone signaling.

Genomic aldosterone actions involve binding of the steroid to its intracellular receptor and translocation of the ligand-receptor-complex to the nucleus, followed by modulation of transcriptional and translational processes. The receptors which transmit these effects have been cloned and represent the superfamily of steroid receptors including the classic, intracellular MR (1, 2).

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In addition to these genomic steroid actions, rapid nongenomic aldosterone effects have been demonstrated. In contrast to genomic aldosterone action, these effects are detected within seconds to few minutes and are not blocked by the classical aldosterone receptor antagonist spironolactone and inhibitors of transcription and protein synthesis, suggesting that they are independent of the MR (3). Rapid effects of aldosterone involve second messengers such as intracellular calcium and cAMP, diacylglycerol and inositoltrisphosphat in various cells of different species (4–10). Specific high affinity binding sites for radioactive aldosterone have been characterized in membranes of different cells (11–14). These binding sites probably represent membrane bound receptors (15). It is unclear if these binding sites are modified classical MRs, as postulated for certain rapid effects of estrogen (16, 17) or if they represent an unrelated receptor protein (18). In the case of aldosterone, the latter hypothesis is supported by several features including the observation that hydrocortisone shows rapid effects at 1000–10000 fold higher concentrations only (7), which is not compatible with binding properties of the intracellular MR (19).

Recently, MR deficient mice have been generated, which show the typical symptoms of pseudohypoaldosteronism (20). They were used to test whether or not rapid nongenomic aldosterone effects can be detected in the absence of the classic intracellular MR.

METHODS

Generation of MR−/− mice. MR knockout mice (MR−/− mice) were generated as previously described (20).

Culture of skin cells. 6–7 days old mice were killed by decapitation. The skin was cut into small pieces and transferred to a culture dish with 3 ml RPMI medium supplemented with 10% fetal calf serum, 15 mmol/L HEPES, penicillin, streptomycin and amphotericin B under standard conditions (37°C, 5% CO_2). After 5–6 days medium was changed and cells were passaged 1–2 days later. Medium was changed every other day.

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Only early passage cells (passages 2–4) were used for experiments. Skin cells were rapidly growing and spindle-like shaped, and thus, resemble fibroblasts. Immunofluorescence staining was performed with polyclonal primary antibodies directed against vimentin (Santa Cruz, Heidelberg, Germany), cytokeratin (Progen, Heidelberg, Germany) and desmin (Santa Cruz, Heidelberg, Germany) and FITC-labeled secondary antibodies (Daco, Glostrup, Denmark). Staining was positive for cytokeratin but not for vimentin and desmin, a pattern which is typical for cells of epithelial origin.

Measurement of intracellular calcium $(Ca^{2+})_i$. Cells were grown on 22 mm cover slips and used 6–7 days after seeding. Before experiments cells were growth arrested by serum deprivation for at least 24 hours.

Imaging of free intracellular calcium $(Ca^{2+})_i$ was performed in single cells. Skin cells were washed two times with 2 ml physiological salt solution (PSS: 135 mmol/l NaCl, 5 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, 10 mmol/l HEPES, 5.5 mmol/l glucose, pH 7.4) to remove medium. Cells were loaded with 5 μmol/L Fura2-AM for 30 min at 37°C, washed with PSS-buffer and placed in a temperature controlled chamber (37°C, Life Science Resources Ltd., Cambridge, UK) holding 0.45 ml of incubation fluid. Cell imaging of $(Ca^{2+})_i$ was performed using a Till Photonics dual wavelength imaging system (Till Photonics GmbH, Gräfelfing, Germany) attached to a Zeiss Aviovert 35 (Zeiss, Hanau, Germany) inverted fluorescence microscope. The imaging camera was an AE2000 system from General Scanning GmbH (Planegg, Germany). A dichroic mirror (Zeiss, Hanau, Germany) separated the excitation wavelength at 340 and 380 nm and emitted light was collected at 510 nm. Integration time was 0.1 sec at an excitation wavelength of 340 nm and 0.06 sec at 380 nm, with a time increment between 6 and 10 sec. Autofluorescence was determined in each experiment by the addition of 5 μmol/l ionomycin and 0.1 mol/l $MnCl_2$ to quench intracellularly located dye. The autofluorescence level was subtracted from each reading before calculation of $(Ca^{2+})_i$. Angiotensin II and thrombin served as positive controls for cell stimulation. The system was calibrated by the method of Grynkiewicz *et al.* (21). Baseline stability was checked for 1–2 min. At times indicated, the hormone stimuli (50 μL) were added. Vehicle alone (ethanol 10^{-8} mol/l) served as a negative control and was without effect on $(Ca^{2+})_i$. $(Ca^{2+})_i$ was analyzed on serial images in regions of interest (ROI) of the cell.

Measurement of intracellular cAMP. Experiments were performed 4–5 days after cell splitting at approximately 80% confluence and after at least 24 h deprivation of serum. First medium was replaced by physiological salt solution (PSS: 135 mmol/l NaCl, 5 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, 10 mmol/l HEPES, 5.5 mmol/l glucose, pH 7.4) containing 500 μmol/l IBMX (3-Isobutyl-1-methylxanthine). After 30 min, isoproterenol, aldosterone or 0.01% ethanol, used as a vehicle, were added. The reaction was stopped at times indicated by aspiration of the medium and by transfering the dishes on ice. Determination of the intracellular cAMP levels was conducted using a commercial radioimmunoassay (Amersham, Braunschweig, Germany) after ethanol extraction.

Statistical analysis. Values are expressed as mean ± SE. For statistical comparison the Friedman's analysis (for multiple comparisons), and the Wilcoxon test for single comparison within one group were used (StatView SE+ Graphics for Apple MacIntosh). For comparison between two groups the Mann-Whitney-U test for unpaired data was applied. The Chi Square test was used as indicated. P-values < 0.05 were considered significant. If appropriate, the Bonferroni correction was applied.

RESULTS

Effects of Aldosterone on Intracellular Calcium Levels

Measurements of intracellular calcium were performed in the wild-type mice cells and in cells from MR knockout mice after stimulation with aldosterone, and angiotensin II or thrombin as positive controls. The basal values of $(Ca^{2+})_i$ were 157 ± 11 nmol/L in the wild-type mice cells versus 158 ± 17 nmol/L in knockout MR cells (no significant difference). Under all conditions, basal $(Ca^{2+})_i$ ranged from ~100 to 200 nmol/L (Figs. 1a–1d).

Addition of 10 nmol/L aldosterone leads to a moderate but significant increase of $(Ca^{2+})_i$, which was 51 ± 7 nmol/L in wild-type cells versus 98 ± 30 nmol/L in cells from mutants. Both increases are significant versus unstimulated baseline levels (Wilcoxon Test: $p < 0.0001$; $n = 29$ cells rsp. $n = 20$ cells). In Figs. 1a and 1b, responses of free intracellular calcium $(Ca^{2+})_i$ after addition of 10 nmol/L aldosterone are shown for cells from wild-type and MR knockout mice. The absolute increases of intracellular calcium were not significantly different between wild-type cells and MR knockout cells. Considering an aldosterone-induced increase $(Ca^{2+})_i$ by at least 10 nmol/L as a positive response (22), 28 out of 29 of the wild-type cells were responsive compared to 19 out of 20 knockout mice cells (Chi Square test: not significant). The response of $(Ca^{2+})_i$ to 10 nmol/L aldosterone was rapid (delayed by less than 60 sec) and thus typical for nongenomic aldosterone action. For wild-type mice cells, a maximum was obtained within 1–2 min, but no plateau was seen as was the case for about half of the knock-out mice cells. The levels of $(Ca^{2+})_i$ 160 sec after application of aldosterone were not significantly different between wild-type and MR knockout ($n = 8$ cells). All cells responded to 1 μmol/L angiotensin II and thrombin 1 U/ml, added to test cell viability (not shown). No differences were seen in the response to angiotensin II and thrombin between wild-type and knockout mice cells (not shown).

For the abundantly available wild-type mice cells, pharmacological features of rapid aldosterone effects were determined for comparison with those obtained in other cells. $(Ca^{2+})_i$ increased from 155 ± 11 nmol/L (basal) to 202 ± 9 nmol/L (Wilcoxon: $p = 0.005$; $n = 10$ cells), despite pretreatment with 10 μmol/L spironolactose for 30 min (Fig. 1c), showing no significant difference to the wild-type cells, which were not preincubated with spironolactone (Fig. 1a).

Neither stimulation with 10 nmol/L nor with 1 μmol/L hydrocortisone showed a significant increase of intracellular calcium in wild-type cells (Fig. 1d).

Effects of Aldosterone on cAMP Levels

Basal cAMP level was 21.9 ± 5.6 pmol/10^6 cells of wild-type mice versus 29.7 ± 7.5 pmol/10^6 cells of knockout mice (not significant). Incubation of the cells with aldosterone (10 nmol/L) increased intracellular cAMP levels 2.2 fold within 1 min in wild-type cells (Fig. 2; $p < 0.02$, $n = 12$) and 10.6 fold for knockout mice cells ($n = 8$, $p < 0.02$ versus basal level, and $p <$

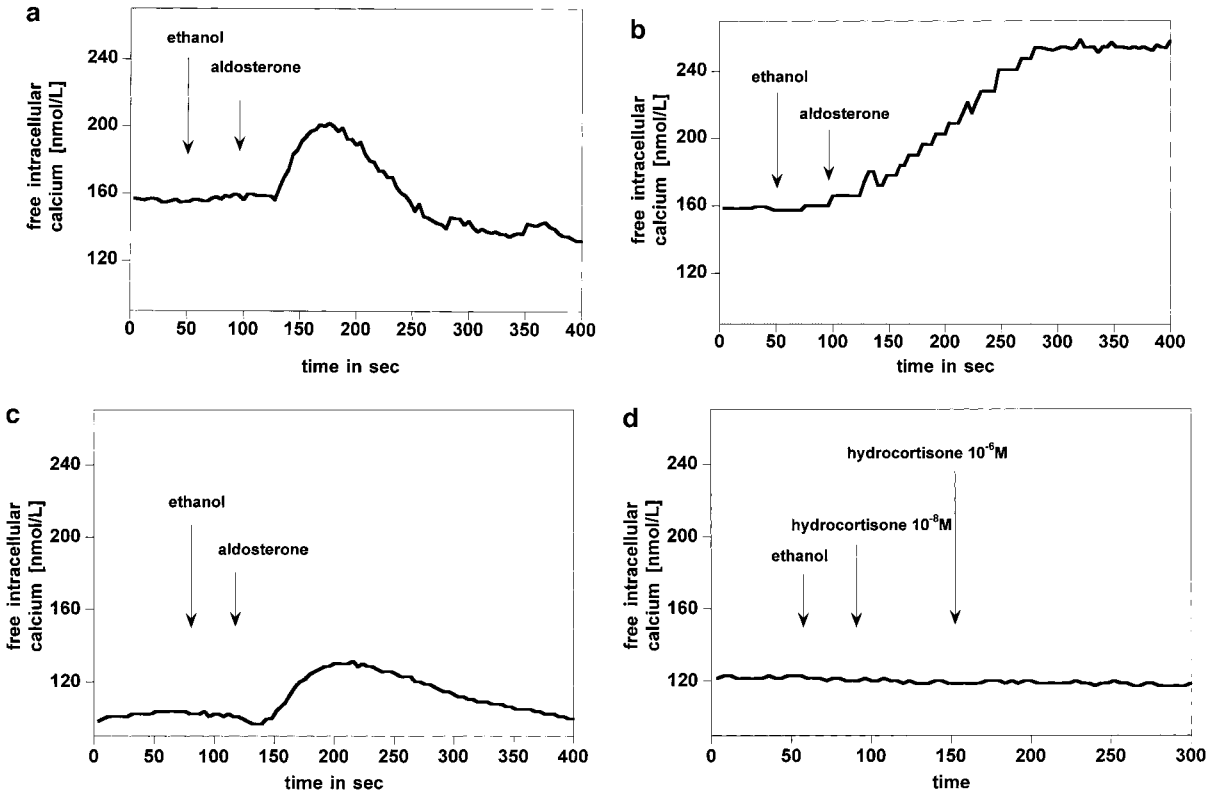

FIG. 1. Time course of free intracellular calcium in skin cells of wild-type mice (a) or MR knockout mice (b), after addition of 0.01% ethanol and 10 nmol/L aldosterone. Wild-type cells were preincubated with spironolactone for 30 min (c). Instead of aldosterone, hydrocortisone 10 nmol/L and 1 μmol/L was applied to wild-type cells in (d).

0.01 versus wild-type). In Fig. 2 the time course of cAMP concentration is shown after stimulation with aldosterone and ethanol as the vehicle for wild-type and MR knockout mice cells. Stimulation of cells with isoproterenol (10 μmol/L), a beta-adrenoceptor stimulator, induced a time-dependent, 96 fold increase of intracellular cAMP levels ($p < 0.02$, $n = 10$; not shown). Basal cAMP values were not affected by addition of the vehicle to the incubation medium (Fig. 2).

DISCUSSION

The main findings of the present study are the following:

(1) Aldosterone (10 nmol/L) increases intracellular levels of calcium and cAMP in skin cells from newborn mice within one minute.

(2) These rapid effects of aldosterone are also seen in mineralocorticoid receptor knockout mice cells.

(3) They expose pharmacological properties typical for rapid, nongenomic effects of aldosterone seen elsewhere (effects not induced by micromolar concentrations of hydrocortisone, not blocked by spironolactone)

After stimulation of skin cells with 10 nmol/L aldosterone, a rapid increase of intracellular calcium and cAMP was found. These results are in agreement with previous studies on rapid aldosterone actions in various cell types (e.g. vascular smooth muscle cells, human mononuclear lymphocytes, porcine endothelial cells, kidney cells and colonic cells) (19, 23). Rapid aldosterone effects on cell signaling including intracellular cAMP, calcium, and phosphoinositide hydrolysis

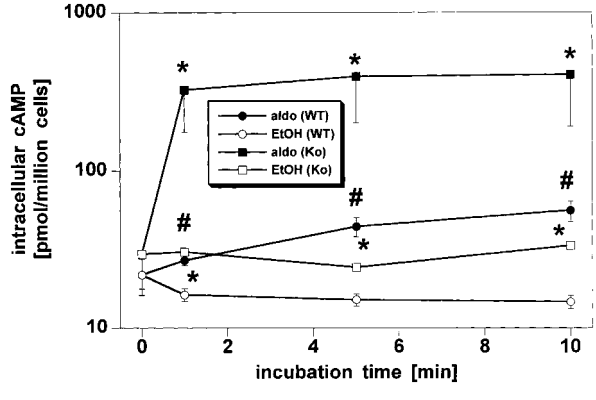

FIG. 2. Intracellular cAMP in skin cells of wild-type (WT) and MR receptor knockout (Ko) mice after stimulation with 0.01% ethanol (EtOH) and aldosterone 10 nmol/L (aldo). Values are expressed as mean ± SE; $n = 12$ (WT), $n = 8$ (Ko). *$p < 0.02$ vs. basal level. †$p <$ 0.01 MR knockout vs. wild-type cells.

occur at physiological concentrations of aldosterone within seconds to few minutes. They are not inhibited by spironolactone, the classic MR antagonist, and not induced by submicromolar concentrations of cortisol. They, therefore, are imcompatible with an involvement of the traditional genomic pathway including the cloned intracellular MR.

These effects are termed "nongenomic", as they are rapid and not blocked by inhibitors of transcription and protein synthesis (5, 7, 19). We have chosen skin cells as an easily accessible model in newborn mice, as smaller organs including heart and vessels are difficult to be obtained in the MR knockout mice which die within 10 days after birth (20).

The present data confirm and prove evidence that rapid aldosterone effects is transmitted by novel membrane receptors for steroids, which are unrelated to the classic intracellular receptors (12, 24).

Radioactive binding studies in membrane preparations from human mononuclear leukocytes, porcine kidney and porcine liver have suggested a binding site compatible with major features of nongenomic aldosterone action (altered specificity, low affinity to spironolactone and cortisol). Important kinetic and pharmacological properties of these aldosterone membrane binding sites, the rapid aldosterone effects on electrolyte transport, cell volume and intracellular second messengers in those cells are very similar; thus, it is fair to assume that these membrane binding sites may represent the receptor which mediates rapid aldosterone effects. These putative receptors are distinct from the classical MR, with its non-selectivity for aldosterone, cortisol and canrenone and the lower affinity for aldosterone (K_d value $\sim$ 2 nmol/L) compared with the affinity of the membrane binding sites (0.1 nmol/L). In contrast, several studies have shown that hydrocortisone, as a glucocorticoid, does not induce the rapid increase of intracellular messengers (e.g. calcium and cAMP) in physiological concentrations (7, 25), a finding which is confirmed in the present study.

As nongenomic steroid action becomes more widely studied, it has been shown, that non-genomic steroids effects may not only be transmitted by unknown membrane receptors, but also by the classic intracellular steroid receptors. Studies on rapid estrogen actions suggest that a modified classical estrogen receptor located in the cell membrane may be involved. Experiments were performed in COS-7 cells to determine the effect of enhanced estrogen receptor expression on the acute response of nitric oxide synthase to estradiol-17β. It was found that the rapid response was augmented four- to fivefold in cells transfected with estrogen receptor compared to sham-transfected cells (26). In addition, the hypothesis of an involvement of a membrane bound, modified estrogen receptor in rapid action is supported by Shaul *et al.* (17), who demonstrated an increase of estradiol-induced rapid endothelial cell NO-synthase activity after transient overexpression of estrogen receptor alpha in ovine fetal pulmonary artery endothelial cells.

Pappas *et al.* (16) stained an estrogen receptor at the membrane level in GH3/B6 pituitary tumor cells by immunocytochemistry, using specific antibodies raised against a peptide representing unique epitopes of the intracellular estrogen receptor.

Opposed to our studies on aldosterone, including the present one, in which the classic mineralocorticoid antagonist spironolactone was ineffective, these effects are blocked by ICI 182,780, a classic antagonist of the cytosolic estrogen receptor.

Similar findings have been reported for glucocorticoids: A glucocorticoid receptor-like antigen was detected in membranes of human lymphoma cells by antibodies (27). As this approach utilized epitopes of the classical, intracellular glucocorticoid receptor (type II) for the immunostaining of steroid membrane receptors, a close relationship if not identity between those types of receptors has been assumed.

In contrast, our findings show that this model of modified classical receptors in the membrane is not applicable to rapid aldosterone effects, because those effects are also seen in MR knockout mice. These findings strongly support the assumption that a novel receptor for aldosterone exists which is clearly distinct from the classical MR. This receptor is membrane-bound, as membrane binding sites for aldosterone have already been identified (14). Cloning of this novel aldosterone membrane receptor represents a major challenge for further research in this area. Aldosterone effects in cells from MR knockout mice on cAMP levels are significantly larger than in those from wild-type mice. A possible explanation may be that the genomic effector suppresses the nongenomic pathway by unknown mechanisms. Other putative explanations could be different developmental or physiological programming until day 6–7 in MR knockout cells leading to altered acute responses to aldosterone. Alternatively, the nongenomic effector may be upregulated in the absence of the genomic pathway. In this context, it is important to note that in all systems investigated for aldosterone action so far, intracellular events (e.g. increases of Na, K, Ca in human mononuclear lymphocytes and vascular smooth muscle cells) of both nongenomic and genomic origin are similar in the directions of changes, but different in their extent. Acute nongenomic rises of calcium are small (50%) whereas late genomically induced increases are large (severalfold), but both modes of action raise calcium. Therefore, cells could utilize the nongenomic pathway more avidly in a compensatory manner, if the genomic receptors are absent.

In conclusion, physiological concentrations of aldosterone rapidly increases intracellular calcium and cAMP levels in MR knockout mice within one minute.

These data prove that the nongenomic aldosterone receptor is clearly distinct from the classical MR. Development of inhibitors, which block both, rapid nongenomic, and delayed genomic steroid effects, may possibly influence future treatment strategies for cardiovascular diseases, such as hypertension and congestive heart failure.

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