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Mineralocorticoid receptor antagonists do not block rapid ERK activation by aldosterone

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Abstract

Aldosterone can elicit rapid nongenomic effects both in vivo and in vitro, often mediated by signal transduction cascades. However, it is not understood how these rapid effects are initiated. In this study we show that aldosterone leads to rapid activation of mitogen activated protein kinases ERK1/2 in the cortical collecting duct cell line M-1. Inhibitors of transcription and translation could not block this activation, which suggests an extranuclear (nongenomic) mechanism. Although it is known that M-1 cells do not contain a transcriptionally functional MR, it is not known whether a closely related protein still could mediate the effects, or an unrelated nonclassic receptor. To test this hypothesis, the effects of four classical mineralocorticoid receptor antagonists were studied. None of the compounds could block the response to aldosterone. Altogether, the data suggest that rapid aldosterone effects in M-1 cells are initiated by a receptor different from the classical mineralocorticoid receptor.

Keywords: Mineralocorticoid receptor antagonists; Nongenomic; Collecting duct; Spironolactone; ERK; MAPkinase; Rapid responses

Aldosterone is a major regulator of ionic homeostasis by modulating cation transport, mainly in kidney and colon. For a long time it was assumed that aldosterone can only act via the classic genomic mechanism involving binding to the intracellular receptor, the mineralocorticoid receptor (MR), thereby affecting transcription and protein synthesis [1]. Yet, many experiments have shown that aldosterone also elicits nongenomic responses by affecting signal transduction, e.g., an increase of intracellular calcium or pH, generation of inositol-1,4,5-trisphosphate, diacylglycerol, cAMP or activation of protein kinase C in various cells of different species [2]. In contrast to genomic aldosterone action, these effects are detected within seconds to few minutes and are not blocked by inhibitors of transcription and protein synthesis, indicating the independence of these effects from the genome. Recently, we could demonstrate rapid nongenomic effects of aldosterone on intracellular calcium and cAMP concentrations even in cells from MR knockout mice [3]. It is not yet clear how these rapid effects are initiated [4]. Specific high-affinity binding sites in membranes of different cells have been identified using radiolabeled aldosterone [5] and others. Since cells from MR knockout mice still display rapid aldosterone effects, such membrane binding sites very likely represent a novel type of aldosterone receptors. In line with this hypothesis is the fact that insensitivity of rapid effects towards the classical MR antagonist spironolactone has been described in different systems [6–8].

The aim of the present study was to investigate rapid nongenomic effects of aldosterone in M-1 cells, a cell line derived from classical target cells for aldosterone, the cortical collecting duct cells of the kidney [9]. The rapid phosphorylation of mitogen activated kinases (MAPK) ERK1/2 has been shown for several steroid hormones including aldosterone [10–13]. Such rapid aldosterone effects have been demonstrated in the Madin–Darby canine kidney cell line [11] and the signaling cascade has been elucidated; however, the question of the identity of the aldosterone receptor has not been addressed.

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Here we aimed at the investigation of the pharmacological profile of the rapid aldosterone induced ERK1/2 activation in order to collect evidence for the receptor's identity, besides elucidation of the signaling pathway. It is a matter of controversy whether rapid nongenomic effects are mediated by the classical MR, or a closely related protein, or whether they use other, structurally unrelated receptors. Although it is known that M-1 cells do not contain a transcriptionally active MR [14], it still cannot be ruled out that, e.g., a truncated or otherwise modified form of MR mediates the response. Therefore, the effect of spirolactone MR antagonists was studied. Furthermore, closely related open-ring compounds that exhibit low affinity at the MR but have been reported to inhibit nongenomic aldosterone responses in human arteries [15] have been used. Furthermore, the effect of cortisol with and without inhibition of 11β-hydroxysteroid dehydrogenase, as well as blockage of the glucocorticoid receptor, was investigated.

In the signaling pathway, rapid Raf, MEK, and MAPK activation by aldosterone and the action of MEK inhibitors were studied.

Materials and methods

Materials. Aldosterone was purchased from Fluka (Buchs, Switzerland), ethanol was obtained from Merck (Darmstadt, Germany), and actinomycin, cycloheximide, canreonate, spironolactone, and RU 38486 (mifepristone) were from Sigma (Taufkirchen, Germany). MEK inhibitors PD98059 and U0126 were from Cell Signaling Technology (Frankfurt, Germany). Cell culture media and reagents were from Invitrogen (Karlsruhe, Germany). RU 26752 and RU 28318 were kindly provided by Hoechst (now Aventis, Frankfurt/Germany).

Tissue culture. The M-1 cell line is derived from the cortical collecting duct (CCD) microdissected from a mouse transgenic for the early region of SV 40 [strain Tg(SV40E) Bri7] and was purchased from the American Type Culture Collection (ATCC No. CRL-2038). Cells were seeded in 75 cm^2 plastic culture dishes in 10 mL medium containing a 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium (Invitrogen). The medium was supplemented with 10% fetal calf serum, 25 IU/mL penicillin, 25 μg/mL streptomycin, and 2 mM L-glutamine. Cells were incubated at 37 °C in an atmosphere containing 5% CO_2. Subconfluent (90% confluence) cultures were subcultured by rinsing with 10× trypsin–EDTA solution for about 30 s at 37 °C until cells detached. The cell suspension was centrifuged at 1200*g* for 5 min and the supernatant was carefully removed. Two milliliters of fresh medium was added and cells were dispensed into new flasks.

Stimulation and inhibition experiments. Before stimulation experiments, M-1 cells were grown to 90% confluence in 35 mm dishes and were serum starved for 2 h in PSS (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Hepes, and 5.5 mM glucose, pH 7.4). Aldosterone prediluted in PSS from a 10 mM stock in ethanol to 100× final concentration was directly added to PSS, and after gentle swirling, cells were incubated for 5 min unless otherwise indicated. Controls were stimulated with ethanol at corresponding concentration. In case of treatment with inhibitors, appropriate predilutions were directly added to PSS prior to the addition of aldosterone unless indicated otherwise. The preincubation times for the different inhibitors used are indicated in the figure legends. Inhibitor controls contained appropriate ethanol concentration for water insoluble compounds or PSS buffer for water soluble reagents. The Western blots shown are representative for at least three independent experiments.

Analysis of Raf, MEK, and MAPK phosphorylation. After treatment, cells were lysed in sample buffer (50 mM Tris/HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, and 100 mM dithiothreitol), sonicated for 15 s, and heated at 100 °C for 5 min. Insoluble material was removed by centrifugation for 4 min. Cell lysates corresponding to 50 μg protein were separated by 12% SDS–PAGE and transferred to a 0.45 μm nitrocellulose membrane (Protran BA85, Schleicher and Schuell, Dassel, Germany) by electroblotting. After blocking with TBST (50 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) supplemented with 5% nonfat dried milk, the membrane was incubated with primary rabbit antibodies against the phosphorylated or unphosphorylated forms of Raf, MEK1/2, and p44/42 MAP kinase (Cell signaling Technology). Bound primary antibody was detected using horseradish peroxidase-conjugated goat anti-rabbit IgG and visualized by an ECL system (Amersham).

For densitometry, Western blots were scanned and analyzed with Alpha Ease imaging software (Alpha Innotec). Relative densitometry results for phosphorylated MAP kinase signals were corrected for total MAP kinase signals. Mean values and standard deviations of three individual experiments were calculated. Statistical analysis was done using the paired *t* test.

Results

ERK1/2 phosphorylation

By using a specific antibody which recognizes the active, phosphorylated forms of MAPK ERK1/2, we analyzed the activation of ERK1/2 in M-1 cells after treatment with aldosterone by Western immunoblot. To ensure equal loading of samples, an antibody that recognizes both the phosphorylated and non-phosphorylated forms of ERK1/2 was used.

Preliminary time course experiments revealed a maximum effect of aldosterone after 5 min which decreased after 10 min; no effect at all was seen in the time interval of 20–60 min after aldosterone administration (data not shown). Therefore, all further experiments were done with 5 min incubation of the cells with aldosterone. Dose–response experiments yielded maximal ERK1/2 phosphorylation after stimulation with 10^{-9} M (1 nM) aldosterone (Fig. 1). The signal for phosphorylated ERK1/2 after 5 min treatment with 1 nM aldosterone increased by a factor of 1.55 ± 0.14 in

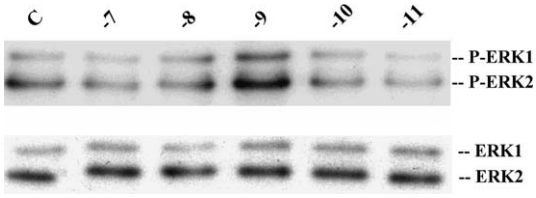

Fig. 1. Dose-dependent activation of ERK1/2 by aldosterone. Western blots containing whole cell lysates from M-1 cells treated for 5 min with vehicle (c, ethanol) or various concentrations of aldosterone (−7, 10^{-7} M, etc.). The upper blots were probed with anti-phospho-ERK1/2 antibody, the lower blots were probed with anti-ERK1/2 antibody.

comparison to control values ($p < 0.05$), whereas a higher concentration of aldosterone (100 nM) led to an increase of only 1.31 ± 0.03 and a lower concentration (0.01 nM) was ineffective (0.95 ± 0.03-fold difference in comparison to control values).

As shown in Fig. 1, both ERK1 and 2 were phosphorylated, which is required for full activation of downstream pathways [16,17].

Effects of actinomycin D and cycloheximide

In the traditional theory of action, steroid hormones are thought to primarily affect nuclear transcription and, subsequently, protein synthesis. To investigate the role of mRNA and protein synthesis in ERK1/2 phosphorylation we preincubated M-1 cells with actinomycin D (5 μg/mL) or cycloheximide (10 μg/mL), respectively, for 15 min. None of them had any detectable effect on the ERK1/2 phosphorylation by aldosterone (Fig. 2). This result indicates that the rapid phosphorylation of ERK1/2 by aldosterone is independent of transcription and translation and, therefore, proceeds by a nongenomic mechanism.

Effects of cortisol and carbenoxolone

It is known that under in vitro conditions, cortisol and aldosterone have similar affinities for MR. However, despite higher circulating levels in vivo, cortisol binding to MR is prevented by 11β-hydroxysteroid dehydrogenase type II (11β-HSD) in aldosterone target tissues [18]. The inhibition of 11β-HSD may result in aldosterone-like action of cortisol by flooding MR.

Cortisol at 100 nM alone had no effect on the ERK1/2 phosphorylation. However, when 11β-HSD was inhibited by 1 μM carbenoxolone, cortisol became an agonist similar to aldosterone (Fig. 3).

Effects of aldosterone antagonists

To investigate whether the classical mineralocorticoid receptor is involved in the upstream events leading to MAPK cascade activation, we used a series of MR antagonists.

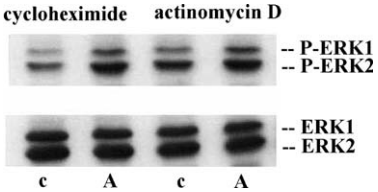

Fig. 2. Effect of cycloheximide and actinomycin D. Western blots from cell lysates pretreated with actinomycin D (5 μg/mL) or cycloheximide (10 μg/mL) for 15 min before stimulation with 10^{-9} M aldosterone (A) or vehicle (c) for 5 min. Blots were probed with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies, respectively.

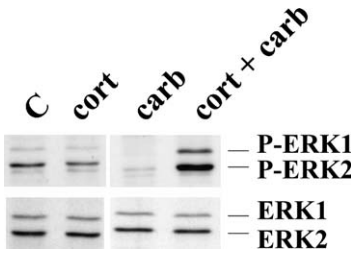

Fig. 3. Effect of cortisol on ERK1/2 phosphorylation. Western blots containing whole cell lysates from M-1 cells treated for 5 min with vehicle (c, ethanol) or cortisol (cort, 10^{-7} M) in the presence or absence of carbenoxolone (carb, 10^{-6} M), added 10 min prior to cortisol. Blots were probed with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies.

Spironolactone, the classic mineralocorticoid antagonist, did not block the effect of 1 nM aldosterone when applied at a 1000-fold excess and added 15 min before aldosterone (Fig. 4A). This indicates that MR might not be involved in the rapid aldosterone mediated ERK1/2 phosphorylation.

RU 26752, a MR antagonist similar to spironolactone with a closed E ring structure, had a small agonist effect by its own on the phosphorylation of ERK 1/2 MAPK. However, the effect of aldosterone after preincubation with RU 26752 remained unchanged compared to the effect of aldosterone without preincubation (Fig. 4A).

The closely related compound RU 28318, which exhibits an open E ring structure, had no effect on ERK1/2 phosphorylation on its own as shown in Fig. 4B. There was no difference between the stimulation with aldosterone alone versus RU 28318 together with aldosterone, either.

The water soluble MR antagonist canrenoate had neither an effect on its own, nor was it able to block the aldosterone induced ERK1/2 phosphorylation.

In order to exclude that aldosterone effects are mediated by the GR, we also applied RU 38486 prior to the stimulation with aldosterone. As shown in Fig. 4A. RU 38486 alone has no effect on phosphorylation of ERK1/2 nor does it blunt the effect of aldosterone. It is therefore unlikely that the ERK1/2 phosphorylation is induced via the GR.

Other signaling events upstream of ERK1/2

Parallel to the rapid aldosterone induced phosphorylation of ERK1/2, we observed phosphorylation of Raf and MEK1/2, the protein kinases upstream of ERK1/2 in the classical MAP kinase cascade (Fig. 5). That aldosterone is using this signaling cascade to elicit its effect was further confirmed by examining the influence of two different MEK inhibitors, i.e., PD-98059 and U0126, on ERK1/2 phosphorylation. Both inhibitors suppressed the aldosterone induced ERK1/2 phosphorylation (Fig. 6).

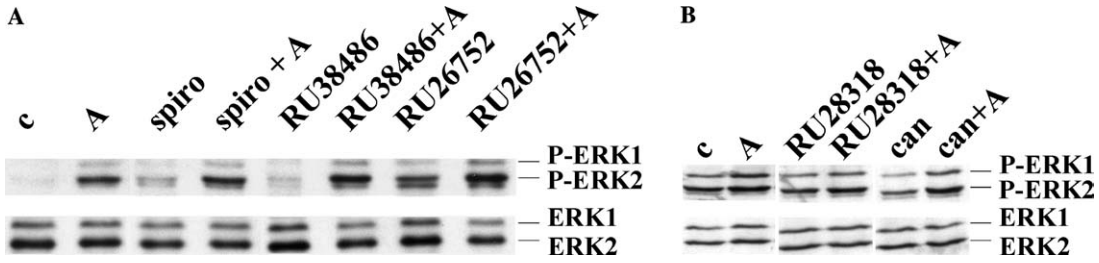

Fig. 4. Sensitivity to MR and GR antagonists of aldosterone induced ERK1/2 activation. Western blots containing whole cell lysates from M-1 cells treated for 5 min with vehicle (c, PBS or ethanol) or aldosterone (10^{-9} M) with or without preincubation (15 min) with antagonists (10^{-6} M). (A) Ethanol soluble antagonists spironolactone (spiro), RU 38486, RU 26752. (B) Water soluble antagonists RU 28318 and canrenoate (can). Blots were probed with anti-phospho-ERK1/2 antibody (upper part) or anti-ERK1/2 (lower part) antibodies. The blots shown are representative for three independent experiments.

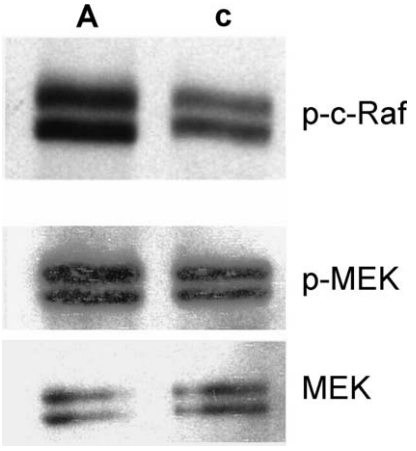

Fig. 5. Activation of Raf and MEK by aldosterone. Western blots containing lysates from cells treated with vehicle (c, ethanol) or aldosterone (A, 10^{-9} M) for 5 min. The blots were probed with anti-phospho-c-Raf, anti-phospho-MEK, and anti-MEK antibodies, respectively.

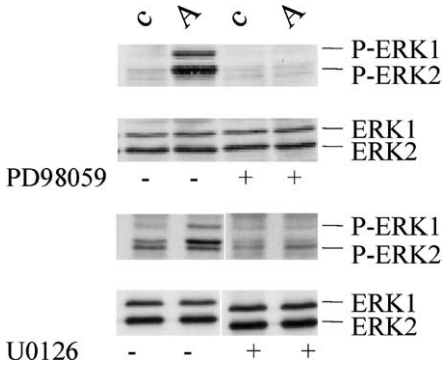

Fig. 6. Inhibition of aldosterone induced ERK1/2 phosphorylation by MEK inhibitors. Western blots containing lysates from M-1 cells treated for 5 min with vehicle (c, ethanol) or aldosterone (A, 10^{-9} M) in the presence or absence of MEK inhibitors PD98059 (10 μM, added 1 h prior to aldosterone) or U0126 (10 μM, added 2 h prior to aldosterone). The blots were probed with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies, respectively.

Discussion

In this study we have demonstrated that the rapid activation of the ERK1/2 MAPK cascade in M-1 cells is not significantly inhibited by a structurally diverse set of antagonists at the classic mineralocorticoid receptor. The cell line used in these studies is derived from murine kidney cortical collecting duct, resembling principal cells which belong to the main targets for aldosterone [19]. In this cell line other rapid aldosterone effects (e.g., increases in intracellular Ca^{2+} levels, pH, and protein kinase C activation) have been described earlier [6]. Here we analyzed phosphorylation of kinases in the MAPK cascade. Studies from other laboratories done with this cell line used confluent cells grown on permeable supports which allowed the cells to form to functionally different sides. In the early stage of this investigation, we compared cells grown this way to cells grown on plastic dishes to 80–90% confluence. As no significant differences were detectable, subsequent experiments were performed on cells grown in plastic dishes.

Our findings of a rapid induction of MAPK phosphorylation which was preceded by MEK and Raf phosphorylation by aldosterone are in line with previous work in smooth muscle cells [7] and the studies of Gekle et al. [11,20] who could show phosphorylation of ERK1/2 and inhibition of this phosphorylation by MEK inhibitors in another kidney cell line, MDCK-C11, resembling intercalated cells of the cortical collecting duct. Gekle et al. [21,22] could furthermore show that aldosterone induced activation of the MAPK cascade involves the EGF receptor. In M-1 cells it remains to be seen whether there is a crosstalk between aldosterone and EGFR signaling. In the *Xenopus laevis* A6 kidney cell line, aldosterone significantly increased MAPK activity as well [23]. However, this aldosterone effect is very different from what we see in M-1 cells, since it became detectable not earlier than 15 min after aldosterone administration with a peak of activation after 2 h and it was a genomic effect dependent on transcription and translation. As the maximum effect was already achieved after 5 min in our experiments, a nongenomic effect can be assumed. This was confirmed by the insensitivity to inhibitors of transcription and translation, which did not block the MAPK activation (Fig. 2).

In order to define events taking place upstream of the MAPK cascade we investigated the pharmacological profile of the MAPK response in order to find evidence for or against involvement of the classic mineralocorticoid receptor in rapid aldosterone signaling.

The classic MR that mediates genomic aldosterone action has roughly equal affinity for cortisol and aldosterone in vitro [18]. However, under in vivo conditions, cortisol is prevented from flooding MR in many tissues by metabolic dehydrogenation to cortisone which has considerably lower affinity. The inhibition of enzyme involved therein, 11β-HSD, therefore results in mineralocorticoid like action of comparatively low doses of cortisol. In our experiments, cortisol at 100-fold higher (100 nM) than the maximally effective aldosterone concentration (1 nM) did not effect any MAPK activation, whereas after inhibition of 11β-HSD by carbenoxolone it became a strong agonist. This behavior resembles the properties of classic MR activation [18]. However, it also may suggest that the receptor mediating the response to cortisol is located within the cell, as 11β-HSD is a cytosolic enzyme. We do not know whether the reponses to cortisol and aldosterone are linked to the same receptor. The closely related steroid corticosterone has been shown to rapidly activate ERK1/2 in a RU 38486 insensitive manner in PC-12 cells [24]. As the response was also elicited by steroid conjugated to BSA, the authors concluded that a membrane receptor was involved.

In order to establish the antagonist selectivity profile, four MR antagonists were tested. Besides spironolactone being the most popular drug, three other selective in vivo MR antagonists were chosen, namely the spironolactone metabolite canreonate and the compounds RU 26752 and RU 28318. None of them could antagonize the effects of aldosterone on ERK1/2 phosphorylation. The classical MR antagonist spironolactone has been shown to be unable to block rapid aldosterone actions in many other studies, although recent work also has identified a system with spironolactone sensitive MAP kinase responses [25]. The compound RU 28318 has been claimed to be an antagonist for nongenomic aldosterone action in rings from uterine arteries as measured by intracellular pH [15]. In contrast to spironolactone, this compound is characterized by an open E ring, a structural component which had been linked to this particular nongenomic antagonist property and that is shared by one of the metabolites of spironolactone, canrenoic acid (for structures, see Fig. 7). Yet, in our study RU 28318 had no effect on nongenomic actions of aldosterone (nor did canrenoate). This is in line with findings of Sato et al. [26] who demonstrated that rapid aldosterone induced suppression of protein kinase C activity in neonatal cardiomyocytes was not influenced by spironolactone nor by RU 28318. Notably, recent electrophysiology studies revealed an antagonist effect of canrenoate on the rapid, aldosterone induced modulation of ion transporter activities [27]. In our experiments canrenoate was inactive.

As all ligands tested here were unable to block the aldosterone response, this is strong evidence against participation of the classic MR in transducing the rapid aldosterone signal. Furthermore, the inefficacy of the classic GR antagonist RU 38486 also rules out GR participation. Another point in disfavor of GR involvement is that aldosterone already shows its effect at a very low concentration (1 nM), while the affinity of

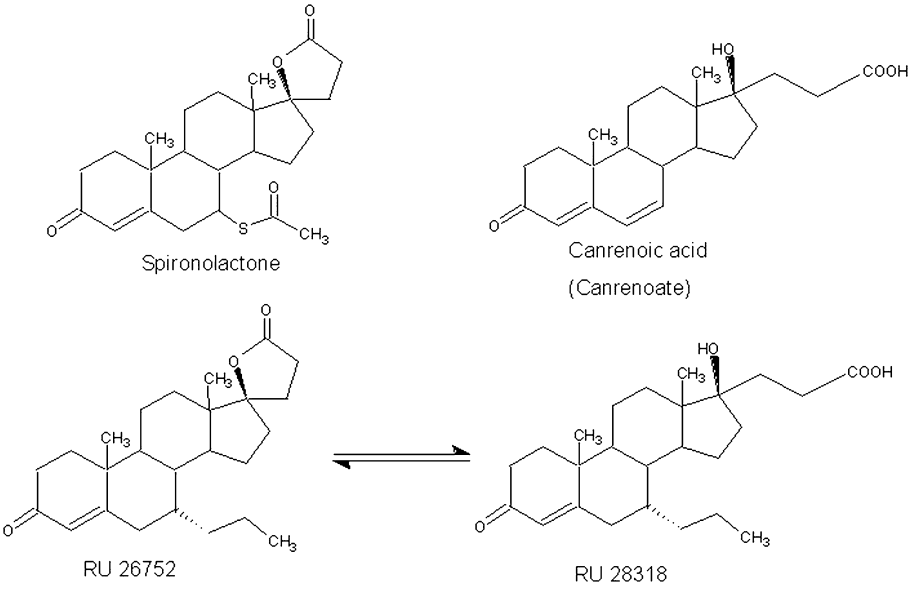

Fig. 7. Structures of MR antagonists used. The compounds with a lactone ring (E ring) on the left side have been reported to exhibit high affinity to the classic MR, while substances with opened E ring (right side) generally have low affinity.

GR for aldosterone is considerably lower, normally requiring aldosterone concentrations in the 100 nM range for receptor activation. However, we have not performed inhibitor experiments for the cortisol/carbenoxolone response; therefore, no conclusion should be drawn about receptor identity in this case.

Generally, it is a matter of controversy, which receptor(s) mediate nongenomic aldosterone action [28]. There is evidence in the literature that M-1 cells do not contain a functional MR [6,14,29], although stimulation of sodium transport by aldosterone and dexamethasone was attributed at least in part to activation of the mineralocorticoid receptor and hence to be inhibitable by spironolactone in one study [30]. In control experiments, aldosterone in the lower nanomolar range was not able to induce sgk expression, although dexamethasone did as judged from RT-PCR experiments (data not shown). However, a truncated or otherwise modified MR still could in principle mediate nongenomic action while being transcriptionally nonfunctional. The ability of cortisol to elicit rapid MAPK phosphorylation after 11β-HSD inhibition could be taken as evidence for such a mechanism. On the other hand, this fact only demonstrates that the receptor (to be defined in this context as any protein that binds aldosterone and relays a signal) also responds to cortisol besides aldosterone, which is not too surprising taking the considerable molecular similarity between these steroids. The enzyme inhibition mechanism is not unique or linked to the classical MR only. If we consider the inability of all classic MR antagonists to block the rapid MAPK induction by aldosterone, the conclusion rather is that a nonclassic receptor is involved.

Membrane aldosterone binding sites have been identified and suggested as the primary target for rapid aldosterone actions [31]. It is unclear whether these membrane binding sites for aldosterone are modified classical mineralocorticoid receptors, in analogy to the well-studied membrane bound estrogen receptors ([32,33]; for review see [34]), or if they represent a new type of membrane receptor. In the case of aldosterone, the latter hypothesis is supported by the data presented here and our recent findings of nongenomic effects of aldosterone on the intracellular concentration of calcium and cAMP in MR knockout mice [3]. It remains elusive how the aldosterone signal is transmitted to the MAPK cascade. Lately, Krug et al. [20] showed that aldosterone upstream of the MAPK cascade rapidly enhances tyrosine phosphorylation of the c-Src and EGFR kinase. We also obtained preliminary data that aldosterone stimulates Src kinase activity in M-1 cells. Yet, the mechanistic link between aldosterone and Src kinase activation is currently missing.

The rapid aldosterone induced ERK1/2 activation has been correlated in kidney cells with rapid activation of Na^+/H^+ exchange [11]. Also in the distal colon, aldosterone induced rapid Na^+/H^+ exchange [35]. A coagonist effect of aldosterone on the action of 1,25-dihydroxyvitamin D_3 action in renal thick ascending limb via ERK has also been reported [36]. Additionally, rapid MAPK activation has been shown to influence gene transcription through phosphorylation of transcription factors or co-factors [37–39]. We have tested during the course of our studies whether putative downstream targets of ERK1/2 were phosphorylated upon aldosterone administration. Yet two classical targets, CREB and p90rsk, were not affected in our system (data not shown). ERK activation, which can be elicited by aldosterone as shown here, or by other agents is involved in renal damage [40] and thus is likely to have a pathophysiological significance.

Rapid effects of aldosterone do play a physiological role with clinical implications, as they were not restricted to isolated cell systems. This has been shown by several clinical studies, e.g., rapid effects of aldosterone on systemic vascular resistance have been demonstrated in healthy male volunteers [41,42]. Moreover, rapid modulation of baroreceptor discharge in dogs [43] and of phosphocreatine levels after isometric muscle contraction in men [44] has been investigated. Though effects of aldosterone alone appear small, synergism with other cardiovascular hormones may be even more important as suggested by data on combined aldosterone/isoproterenol action [45], and data on angiotensin II/aldosterone effects on intracellular calcium concentrations.

We demonstrated that the MAPK pathway is involved in transducing the rapid nongenomic aldosterone signal in the kidney M-1 cell line. The activation of MAPK ERK1/2 by aldosterone was insensitive towards four different antagonists of the MR with either open- or closed-E-ring structures. This strongly suggests that the aldosterone induced activation of the MAPK cascade is initiated by a receptor not closely related to the classical mineralocorticoid receptor. Searching for appropriate inhibitors that are able to antagonize nonclassic aldosterone responses may improve the choice of therapies for pathological conditions with elevated aldosterone levels.

Acknowledgments

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