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Human Mineralocorticoid Receptor Expression Renders Cells Responsive for Nongenotropic Aldosterone Actions

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The steroid hormone aldosterone is important for salt and water homeostasis as well as for pathological tissue modifications in the cardiovascular system and the kidney. The mechanisms of action include a classical genomic pathway, but physiological relevant nongenotropic effects have also been described. Unlike for estrogens or progesterone, the mechanisms for these nongenotropic effects are not well understood, although pharmacological studies suggest a role for the mineralocorticoid receptor (MR). Here we investigated whether the MR contributes to nongenotropic effects. After transfection with human MR, aldosterone induced a rapid and dose-dependent phosphorylation of ERK1/2 and c-Jun NH_2-terminal kinase (JNK) 1/2 kinases in Chinese hamster ovary or human embryonic kidney cells, which was reduced by the MR-antagonist spironolactone and involved cSrc kinase as well as the epidermal growth factor receptor. In primary human aortic endothelial cells, similar results were obtained for ERK1/2 and JNK1/2. Inhibition of MAPK kinase (MEK) kinase but not of protein kinase C prevented the rapid action of aldosterone and also reduced aldosterone-induced transactivation, most probably due to impaired nuclear-cytoplasmic shuttling of MR. Cytosolic Ca^{2+} was increased by aldosterone in mock- and in human MR-transfected cells to the same extend due to Ca^{2+} influx, whereas dexamethasone had virtually no effect. Spironolactone did not prevent the Ca^{2+} response. We conclude that some nongenotropic effects of aldosterone are MR dependent and others are MR independent (*e.g.* Ca^{2+}), indicating a higher degree of complexity of rapid aldosterone signaling. According to this model, we have to distinguish three aldosterone signaling pathways: 1) genomic via MR, 2) nongenotropic via MR, and 3) nongenotropic MR independent. (*Molecular Endocrinology* 19: 1697–1710, 2005)

THE CLASSICAL ACTION of aldosterone involves enhancement of Na^+ reabsorption and thereby long-term regulation of blood pressure (1). Nowadays, it is evident that the importance of aldosterone and the mineralocorticoid receptor (MR) extends beyond this classical action. It has been demonstrated that aldosterone promotes cardiovascular and renal fibrosis due to tissue remodeling as well as endothelial dysfunction, independently of its effects on blood pressure or salt homeostasis (2–6). The pathophysiological role of aldosterone has received impressive support from clinical studies, especially RALES and EPHESUS (7, 8). Low doses of MR antagonists led to a dramatic improvement of mortality in patients with severe congestive heart failure or after myocardial infarction (7, 8). Thus, it is clear that aldosterone and the MR exert important nonclassical, nonepithelial actions. Unfortunately, the underlying mechanisms are only poorly understood.

According to the concept of classical steroid action, aldosterone binds to the cytosolic MR (9), which acts as a transcription factor. Consequently, the expression of various proteins like the epithelial sodium channel, Na^+-K^+-ATPase, the serum- and glucocorticoid-regulated protein kinase and the epidermal growth factor receptor (EGFR) is modulated (9–16). Yet, steroids, including aldosterone, can also induce rapid, nongenotropic responses by interfering with, for example, regulation of intracellular calcium (17–21), protein kinase C (PKC) and ERK1/2 phosphorylation (22–30). Furthermore, there seems to exist a functional cross talk between classical and nongenotropic ac-

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Abbreviations: BIM, Bisindolylmaleimide I; CHO, Chinese hamster ovary; EGFP, enhanced GFP; EGFR, epidermal growth factor receptor; ER, estrogen receptor; GFP, green fluorescent protein; GR, glucocorticoid receptor; GRE, glucocorticoid response element; h, human; HaoECs, human aortic endothelial cells; HEK, human embryonic kidney; JNK, c-Jun NH_2-terminal kinase; MEK, MAPK kinase; MR, mineralocorticoid receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; SEAP, secretory alkaline phosphatase.

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tions (26, 31, 32). It is now clear that the classical receptors for estrogen (ER), progesterone (PR), androgens, and glucocorticoids (GR) contribute to nongenotropic effects (22, 25, 33–40) in many cases via pathways involving ERK1/2 kinases. However, some nongenotropic effects arise from plasma membrane steroid receptors, which are either classical receptors localized at the membrane [*e.g.* ER (23, 24)] or specialized membrane receptors, like murine PR and G protein-coupled receptor 30 (41, 42). For aldosterone, it had been suggested that the alternative actions are mediated by a plasma membrane receptor (25). Yet, this putative receptor has not been identified. The possible role for the MR with respect to nongenotropic effects has not been investigated as extensively as for other steroid receptors. A study with fibroblasts from MR knockout mice showed no difference of the Ca^{2+} response compared with wild-type animals (43), indicating that the MR is not necessary for aldosterone-induced Ca^{2+} signaling. This does not exclude that other signaling pathway are MR dependent. Pharmacological evidence for a physiologically relevant role of the MR in nongenotropic actions was presented, for example, for modulation of plasma membrane Na^+-transporters (26, 44, 45), vascular reactivity and NO synthesis (46–48) or src activity (49). These data show that physiological significant rapid aldosterone effects can be observed in classical target cells (26) as well as in nonclassical tissue known to express the MR (46, 47). Because at least some of the effects were sensitive to MR antagonists, an involvement of the classical MR in rapid signaling is conceivable. The present study investigates the involvement of MR in nongenotropic signaling on the cellular level using heterologous expression systems. This approach was chosen to test the hypothesis using an additional nonpharmacological tool. Our data show that MR contributes to rapid aldosterone-induced activation of the ERK1/2 and c-Jun NH_2-terminal kinase (JNK) 1/2 pathway but not to Ca^{2+} signaling, indicating a higher degree of complexity of rapid aldosterone signaling.

RESULTS

ERK1/2 Phosphorylation in Stable Transfected Chinese Hamster Ovary (CHO) Cells

To determine the possible role of the MR for nongenotropic actions of aldosterone, we generated several clones of CHO cells stably transfected with the human (h) MR (CHO-MR) (15) and compared these clones with cells transfected with control plasmid (mock-transfected cells). Wild-type CHO cells do not express detectable levels of the MR (15). Figure 1, A and B, shows that CHO-MR, but not CHO-mock cells, respond to nanomolar concentrations of aldosterone with a rapid (5 min) and dose-dependent increase of ERK1/2 phosphorylation. The insets show Western blots confirming the ELISA data. Responses to a positive control [1 μmol/liter phorbol 12-myristate 13-acetate (PMA)] were identical in CHO-MR and CHO-mock cells (Fig. 1, C and D). It has been shown that the EGFR can be involved in rapid steroid signaling (24) and that MR enhances EGFR expression and EGF responsiveness (15) (see also Fig. 1, C and D). The EGFR is an aldosterone-induced protein in MR-expressing cells. Thus, when CHO cells are transfected with MR and grown in aldosterone containing medium (*e.g.* medium with serum) EGFR expression in these cells is stimulated. To rule out, that enhanced EGFR-expression alone is sufficient to elicit rapid aldosterone responses, we tested EGFR-transfected CHO cells (15). Although these cells (Fig. 1E) responded to EGF like CHO-MR cells, aldosterone alone was without effect with respect to ERK1/2 phosphorylation, confirming earlier data (50).

Aldosterone induced ERK1/2 phosphorylation in four different CHO-MR cell clones was tested (Fig. 2A). CHO cells transfected with hGR (15) did not respond to aldosterone with ERK1/2 phosphorylation (123 $\pm$ 23% of control, n = 3). The time course of aldosterone-induced ERK1/2 phosphorylation in CHO-MR cells was sustained, in contrast to EGF or PMA (Fig. 2B), which showed a larger but partially transient effect. In a separate experiment we determined ERK1/2 phosphorylation after 24 h. The aldosterone group showed 210 $\pm$ 15% pERK1/2 as compared with controls ($P < 0.05$, n = 3). The MR-antagonist spironolactone (1 μmol/liter; Fig. 2C) inhibited aldosterone-induced ERK1/2 phosphorylation, indicating a direct involvement of MR. Spironolactone *per se* induced a slight but significant increase of ERK1/2 phosphorylation, for which we do not have an explanation. Possibly, spironolactone acts as a partial agonist at the MR. Similar effects were observed for the MR-antagonist RU28318 (data not shown). In summary, comparison of the different CHO-MR cell clones, taken together with the inhibitory action of spironolactone, show that aldosterone induces a rapid, moderate but sustained, MR-dependent phosphorylation of ERK1/2 in our experimental model.

This activation is prevented by the MAPK kinase (MEK) inhibitor U0126 (10 μmol/liter; Fig. 2E), which also prevents EGF-induced ERK1/2 phosphorylation (Fig. 2D). Furthermore, inhibition of the EGFR kinase by 100 nmol/liter AG1478 prevented EGF- as well as aldosterone-induced ERK1/2 phosphorylation (Fig. 2, D and E). A second EGFR kinase inhibitor, AG112 (1 μmol/liter), exerted a similar effect. Because it has been shown that aldosterone leads to a rapid activation of the Src kinase (49), we tested the Src inhibitor PP2 [4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine] (100 nmol/liter). Inhibition of cSrc kinase with PP2 prevented aldosterone-induced ERK1/2 phosphorylation but not EGF-induced ERK1/2 phosphorylation (Fig. 2, D and E). Thus, cSrc seems to be located upstream of EGFR under our experimental conditions. These data indicate that, comparable to estradiol and ER (24), MR uses the cSrc and EGFR signaling pathway to stim-

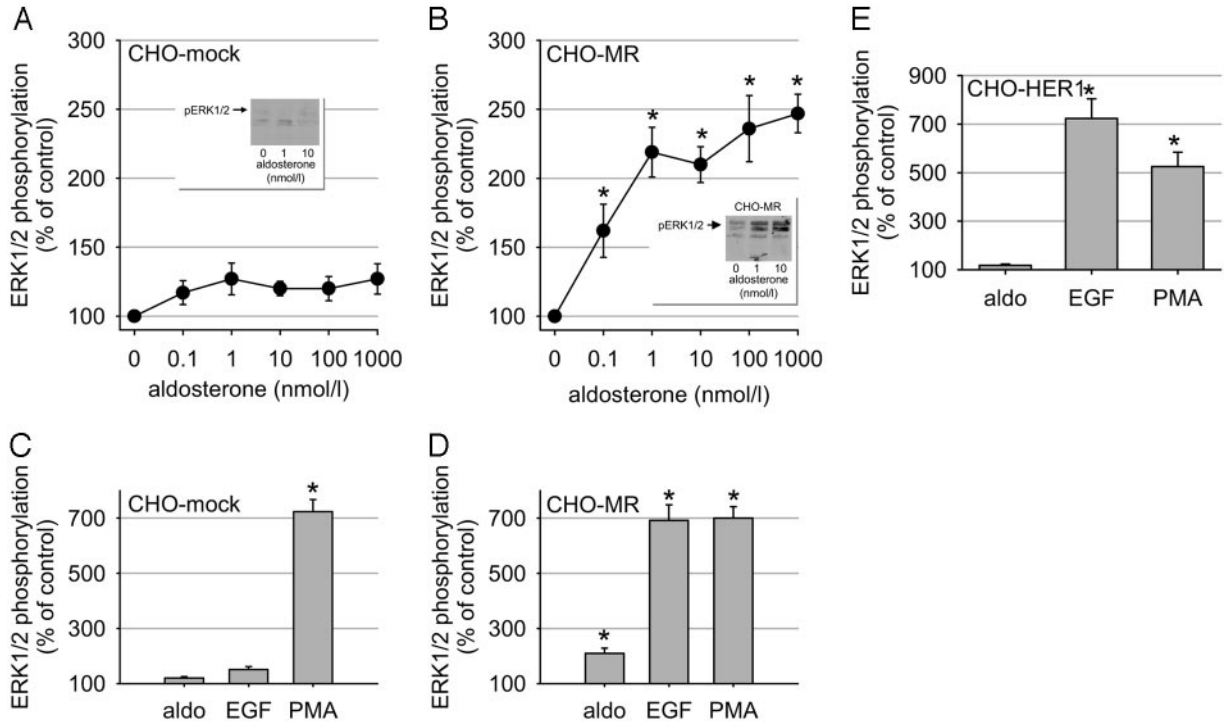

Fig. 1. Aldosterone Induces Rapid ERK1/2 Phosphorylation in Cells Stably Transfected with hMR

A, A 5-min exposure of CHO-mock cells to aldosterone. B, A 5-min exposure of CHO-MR cells to aldosterone. C, A 5-min exposure of CHO-mock cells to 10 nmol/liter aldosterone, 10 μg/liter EGF, or 1 μmol/liter PMA. D, A 5-min exposure of CHO-MR cells to 10 nmol/liter aldosterone, 10 μg/liter EGF, or 1 μmol/liter PMA. E, A 5-min exposure of CHO-HER1 cells (stably transfected with hEGFR) to 10 nmol/liter aldosterone, 10 μg/liter EGF, or 1 μmol/liter PMA. Data in the line/scatter blots were obtained by ELISA. The *insets* show representative Western blots, confirming the ELISA data. n = 12–60 for all line/scatter blots and bar graphs. *, $P < 0.05$ *vs.* respective control.

ulate ERK1/2 phosphorylation. The nature of MR-cSrc interaction is still elusive. In preliminary experiments we tried to coimmunoprecipitate cSrc and MR; however, we have not been successful. One explanation could be a technical failure because the MR is not easy to handle with protein-biochemical techniques. The other explanation is that the two proteins do not interact directly. We will address this question in future studies in more detail, to come to reliable conclusions. However, activation of cSrc could also occur by indirect mechanisms. Only recently cSrc activation via Pyk2 after alteration of cytosolic pH has been described (51). Possibly, aldosterone also acts via an indirect pathway.

As already mentioned, we do know very little with respect to the molecular details underlying these events and future studies will have to shed more light on this particular issue. It has been shown that aldosterone can rapidly modulate PKC activity (20, 25, 26, 45, 53), a signaling pathway capable of activating ERK1/2 (54). We could not detect any effect of PKC inhibition by 100 nmol/liter bisindolylmaleimide I (BIM) (54) on aldosterone- or EGF-induced ERK1/2 phosphorylation (Fig. 2, D and E) under our experimental conditions. Of course, our data do not rule out PKC activation, but they do indicate that this putative activation is not necessary for MR-mediated, aldosterone-induced ERK1/2 phosphorylation.

ERK1/2 Phosphorylation in Transient Transfected Human Embryonic Kidney (HEK) Cells

The use of cells stably transfected with MR has two disadvantages: 1) cell cloning could result in a nonrepresentative selection of certain cell types; and 2) controlling expression at the protein level has proven difficult in CHO cells, due to the low cytoplasm-to-nucleus-ratio and the insufficient quality of available antibodies. Although we circumvented disadvantage 1) by using four independent clones, we tested our hypothesis in a different experimental system, using HEK cells transiently transfected with pEGFP-C1-hMR (55). Expression of EGFP-C1-hMR was verified at the protein level in transfected cells by ELISA (Fig. 3A), fluorescence (Fig. 3B) and Western blot (Fig. 3C). In HEK cells transfected with EGFP-C1, aldosterone did not transactivate a glucocorticoid response element (GRE)-secretory alkaline phosphatase (SEAP) reporter construct, confirming the lack of MR expression functionally (Fig. 4A). After transfection with hMR or EGFP-C1-hMR aldosterone transactivated the GRE-SEAP reporter, as shown in Fig. 4B. The EC_{50} value for aldosterone was approximately 0.3 nmol/liter.

EGFP-C1-hMR-transfected cells responded to aldosterone (10 nmol/liter) with a rapid (5 min) increase of ERK1/2 phosphorylation, whereas EGFP-C1-trans-

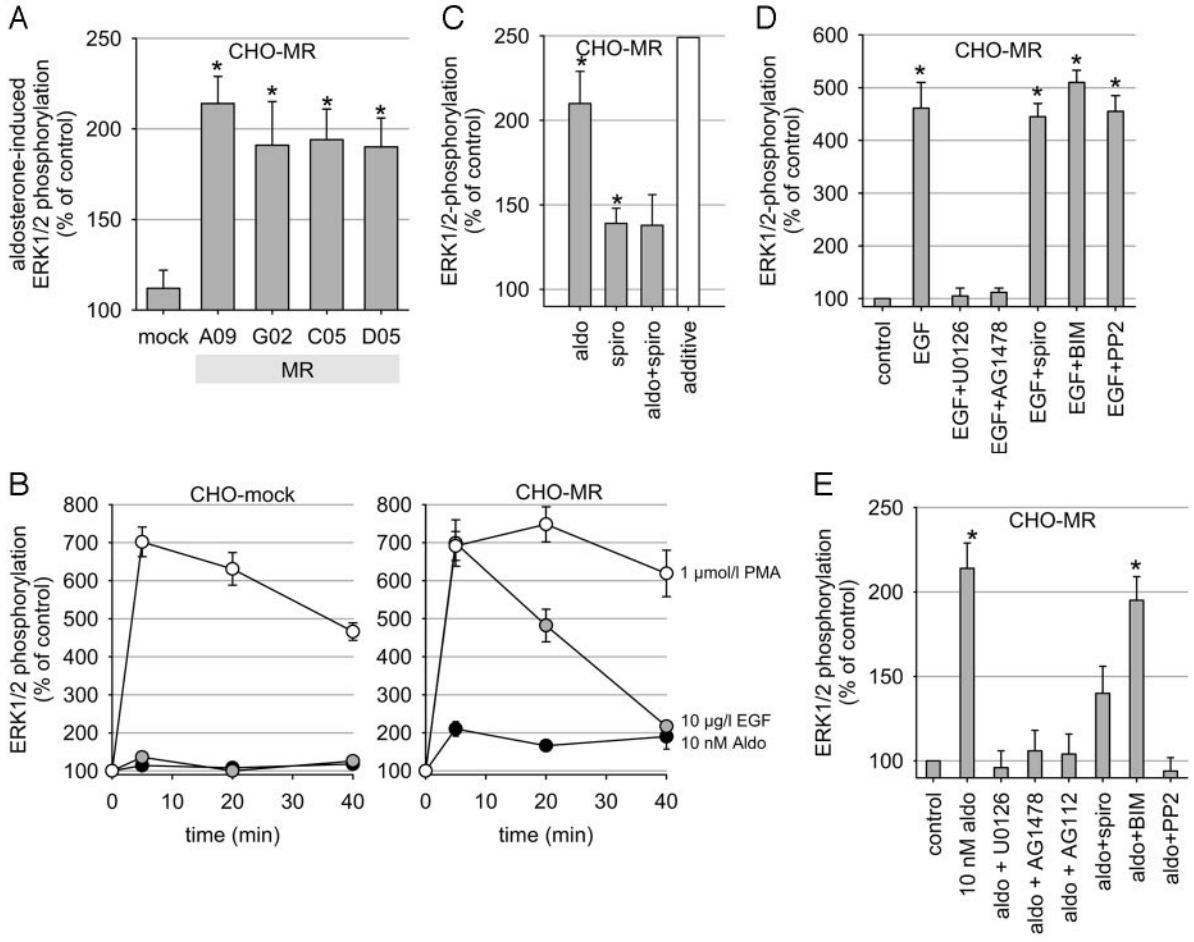

Fig. 2. Aldosterone-Induced Rapid ERK1/2 Phosphorylation

A, A 5-min exposure of four clones of CHO-MR cells to 10 nmol/liter aldosterone. B, Time course of ERK1/2 phosphorylation by aldosterone, EGF and PMA in CHO-mock and CHO-MR cells. C, A 5-min exposure of CHO-MR cells to 10 nmol/liter aldosterone ± 1 µmol/liter of the MR-anatgonist spironolactone (spiro). The *empty bar* shows the expected value when the effects of aldosterone and spironolactone are additive (= no interaction). D, EGF-induced ERK1/2 phosphorylation is inhibited by 10 µmol/liter U0126 (MEK1/2 inhibitor) and 100 nmol/liter AG1478 (EGFR kinase inhibitor) but not by 1 µmol/liter spironolactone, 100 nmol/liter PP2 (cSrc kinase inhibitor), or 100 nmol/liter BIM (PKC inhibitor). E, Aldosterone-induced ERK1/2 phosphorylation is inhibited by 10 µmol/liter U0126 (MEK1/2 inhibitor), 100 nmol/liter AG1478 or 1 µmol/liter AG112 (EGFR kinase inhibitors), and 100 nmol/liter PP2 (cSrc kinase inhibitor) but not by 100 nmol/liter BIM (PKC inhibitor). Exposure time = 5 min. n = 9–60 for all values plotted. *, $P < 0.05$ *vs.* respective control.

fected cells did not respond (Fig. 3D). PMA exerted similar effects under either condition. EGFR kinase inhibition by 100 nmol/liter AG1478 or cSrc kinase inhibition by 100 nmol/liter PP2 prevented the effect of aldosterone in HEK cells, similar to CHO-MR cells (Fig. 3D). Thus, the data obtained in HEK cells support our conclusions drawn from the data obtained with CHO cells. MR is necessary for the rapid aldosterone-induced phosphorylation of ERK1/2 and requires the EGFR as well as cSrc.

Figure 3E shows the phospho-Tyr pattern of EGFP-hMR-expressing cells after exposure to aldosterone for 5 min. The intensity of an approximately 55-kDa band increased in the presence of aldosterone. Most likely this band represents cSrc, which, according to the pharmacological data presented in Figs. 2E and 3D, contributes to aldosterone-induced ERK1/2 phosphorylation. To test this hypothesis further, we detected phosphorylated cSrc using an antibody specific for ^{pTyr416}cSrc. As shown in Fig. 3F, the phospho-cSrc signal increased after exposure to aldosterone (5 min). Finally, we detected phospho-EGFR using an antibody specific for ^{pTyr1086}EGFR. Figure 3G shows that aldosterone induced EGFR phosphorylation (5-min exposure). Because inhibition of cSrc kinase by PP2 did not prevent the effect of EGF (Fig. 2D) on ERK1/2 phosphorylation, we would like to suggest that aldosterone acts via cSrc on EGFR, which finally activates ERK1/2. As already mentioned, we are aware

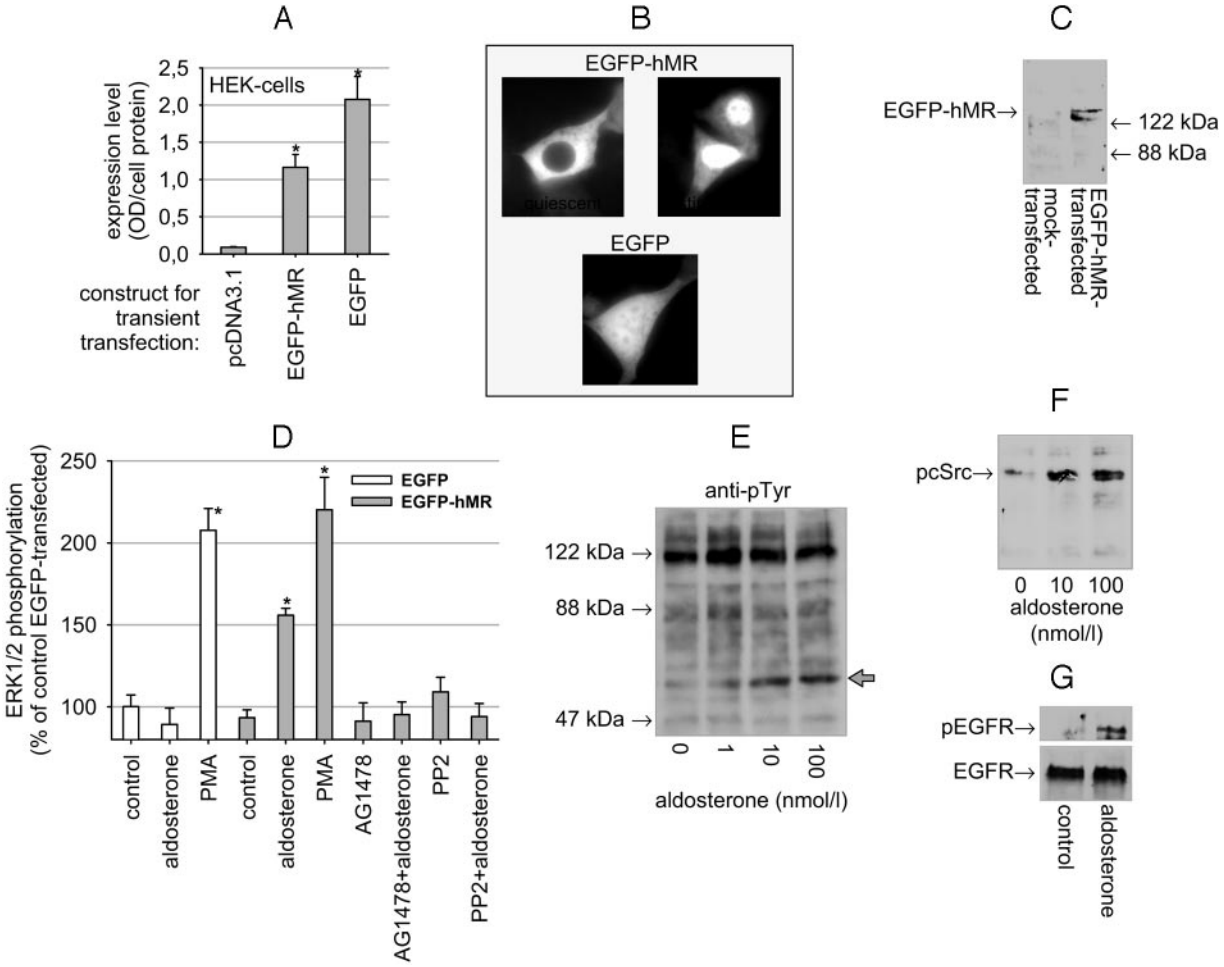

Fig. 3. Aldosterone Induces Rapid ERK1/2 Phosphorylation in HEK Cells Transiently Transfected with hMR Fused to EGFP (EGFP-hMR)

A, Determination of EGFP-hMR and EGFP expression by ELISA. n = 6. B, Determination of EGFP-hMR and EGFP expression by EGFP fluorescence (magnification, ×100). EGFP-hMR distribution depends to MR stimulation. Unstimulated receptors localize to the cytoplasm whereas stimulated receptors localize to the nucleus. C, Determination of EGFP-hMR expression by immunoblot. D, ERK1/2 phosphorylation after a 5-min exposure. Aldosterone (10 nmol/liter) was effective only in EGFP-hMR-transfected cells. PMA (1 μmol/liter) exerted similar effects in EGFP-hMR- and in EGFP-transfected cells. Inhibition of EGFR kinase by 100 nmol/liter AG1478 or cSrc kinase by 100 nmol/liter PP abolished the effect of aldosterone. n = 9. E, A 5-min exposure to aldosterone leads to enhanced Tyr phosphorylation of an approximately 55-kDa protein (*arrow*, the experiment was performed twice with similar results). F, A 5-min exposure to aldosterone leads to enhanced Tyr phosphorylation of cSrc (corresponding to the ~55-kDa protein). G, A 5-min exposure to aldosterone leads to enhanced Tyr phosphorylation of EGFR.

that this hypothesis requires further detailed investigation in future studies.

A cross talk between rapid actions of steroids and their classical pathway has been proposed, *e.g.* with respect to ERK1/2 (56) and PKC (26). ERK1/2 stimulates PR transactivation activity (56) but can inhibit glucocorticoid receptor transactivation activity in certain cell types (57). In a first approach, we tested the effect of U0126 on MR-dependent, aldosterone-induced transactivation. As shown in Fig. 4C, U0126 reduced transactivation in a dose-dependent manner. Although the underlying mechanisms for altered MR-induced transactivation are not yet known, there is the possibility that MR-mediated transactivation may be regulated differently by MAPK as compared with GR-mediated transactivation, which may be either inhibited or stimulated (57, 58). To test this hypothesis, we investigated the effect of U0126 in cells transfected with hGR and stimulated with 100 nmol/liter dexamethasone. However, under our experimental conditions U0126 also reduced GR-mediated transactivation activity in HEK cells (Fig. 4D), although the sensitivity seems to be lower as compared with MR. Half-maximum inhibition of GR-mediated transactivation was achieved with U0126 concentrations approximately five times higher as compared with MR. Possibly, ERK1/2 act on more than one site during receptor activation (*e.g.* nuclear shuttling and coactivator stimulation) or different isoforms of MAPK are involved. To exclude nonspecific effects of U0126 on

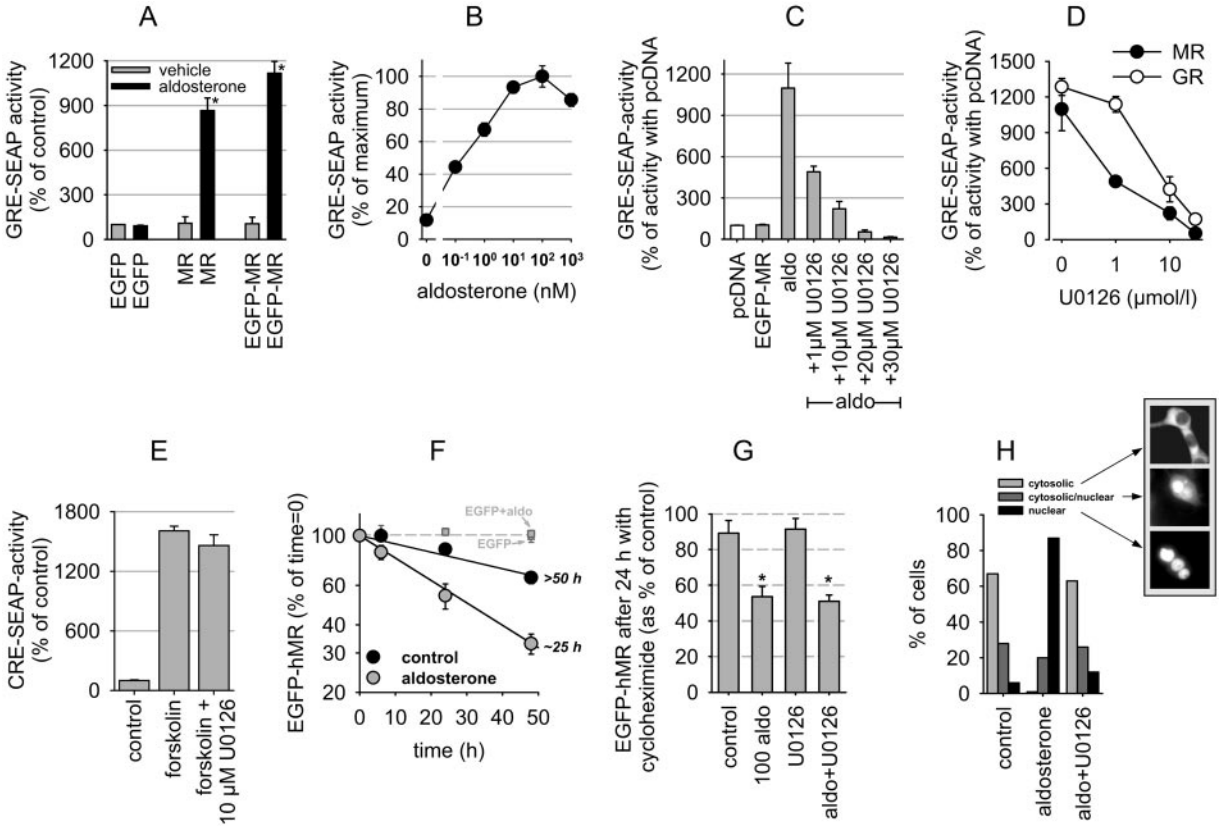

Fig. 4. Interaction of hMR with ERK1/2

A, Stimulation of GRE-dependent transactivation was determined with a GRE-SEAP reporter construct. Cells transfected with EGFP alone did not respond to aldosterone (100 nmol/liter, determined after 24 h). B, In cells transfected with hMR or the EGFP-hMR-construct aldosterone induced transactivation in a concentration-dependent manner. n = 12. C, Aldosterone-induced (100 nmol/liter) stimulation of GRE-dependent transactivation was reduced by U0126, an inhibitor of ERK1/2 activation. n = 9. *, $P < 0.05$ *vs.* respective control. D, In cells transfected with hGR and stimulated with 100 nmol/liter dexamethasone U0126 also inhibited transactivation. However GR-mediated transactivation seems to be less sensitive because higher concentrations of U0126 were required. n = 6–9. E, Forskolin-induced (10 μmol/liter) stimulation of CRE-dependent transactivation was not reduced by U0126. n = 3. F, Degradation of EGFP-hMR is accelerated by aldosterone (100 nmol/liter). EGFP is degraded much slower as compared with EGFP-hMR and the rate of degradation is not affected by aldosterone. n = 6. Degradation was determined in the presence of the protein synthesis inhibitor cycloheximide (10 μmol/liter). G, Comparison of EGFP-hMR degradation over 24 h. Aldosterone-induced degradation was not prevented by U0126. Aldosterone (100 nmol/liter). U0126 (10 μmol/liter). n = 6. *, $P < 0.05$ *vs.* control. H, Distribution of EGFP-hMR in HEK cells. For each experimental condition, 300 cells were evaluated. Cells were distributed into three categories as shown by the examples in the *inset*: only cytosolic appearance of EGFP-hMR (*cytosolic*), cytosolic and nuclear appearance (*cytosolic/nuclear*), only nuclear appearance (*nuclear*). Aldosterone (100 nmol/liter) induced cytosolic-to-nuclear shuttling and in approximately 90% of the cells MR showed nuclear localization. This shuttling was prevented by U0126.

SEAP-reporter constructs, we determined forskolin-induced activation of CREB using a CRE-SEAP reporter. As shown in Fig. 4E, U0126 did not interfere with CREB. Thus, U0126 seems not to act via a non-specific mechanism.

In summary, the data suggest that there may be a positive cross talk between nongenotropic ERK1/2 activation and aldosterone-mediated regulation of gene expression, at least in certain cell types. Of course, the mechanisms of this cross talk have to be investigated in more detail in the future. Two possibilities are the modulation of MR degradation or of MR shuttling into the nucleus. We investigated MR degradation by ELISA as described in *Materials and Methods*. Figure 4F shows the decline of MR expression over time when protein synthesis was inhibited by cycloheximide. Under control conditions (in serum- and hormone-free media), MR expression declined slowly with an apparent half-life greater than 50 h. In the presence of aldosterone, degradation was accelerated and the apparent half-life was approximately 25 h. These data are in agreement with the hypothesis that activated steroid receptors undergo enhanced degradation (56, 59, 60). EGFP [enhanced green fluorescent protein (GFP)] expression declined much slower over time as compared with MR, and this decline was not influenced by aldosterone, demonstrating the specificity of the observed effects. Next, we compared MR expres-

sion after 24 h in the presence and absence of U0126 (Fig. 4G). Aldosterone-induced decline of MR expression was not affected by U0126, indicating that ERK1/2 do not act on MR transactivation via alteration of MR half-life. These results also suggest that aldosterone-induced activation of MR is not affected by U0126 because mainly activated receptors are supposed to undergo proteasomal degradation. In contrast, the appearance of EGFP-C1-MR in the nucleus during aldosterone exposure was inhibited by U0126 (Fig. 4H). Thus, ERK1/2 seems to be involved in MR shuttling to the nucleus. Consequently, when ERK1/2 phosphorylation is prevented, activated MR will not enter the nucleus but will be directly delivered to degradation. The precise mechanisms underlying the observed effects are currently still elusive. We do not know whether MR is phosphorylated by MAPK as has been described for other steroid receptors (24, 60–62). Recently, aldosterone-induced serine phosphorylation of MR has been described (26), furthermore hMR contains a putative ERK1/2 phosphorylation site (PQSP at position 701). Therefore, ERK1/2-dependent phosphorylation is conceivable but has to be addressed in detail in future studies. Of course, we also have to consider the possibility that MAPK interfere with MR function by additional, indirect, pathways, like phosphorylation of coactivators (63) or other transcription factors which could exert transcriptional synergy (61, 64).

JNK1/2 and p38 Phosphorylation

To test whether the MR is involved in further aldosterone-mediated rapid signaling events, we investigated JNK1/2 and p38 phosphorylation, two other pathophysiological relevant MAPKs (65), as well as cytosolic Ca^{2+}-homeostasis. Figure 5, A and B, shows that JNK1/2 phosphorylation was stimulated by aldosterone in CHO-MR cells but not in controls. The insets show Western blots confirming the ELISA data. Responses to a positive control [1 μmol/liter anisomycin (66)] were identical in CHO-MR and CHO-mock cells (Fig. 5, C and D). We also tested the effect of EGF on JNK1/2 phosphorylation. CHO-MR, but not CHO-mock cells showed a response (Fig. 5, C and D). The time courses of EGF- or aldosterone-induced JNK1/2 phosphorylation are similar (Fig. 5E), in contrast to ERK1/2 phosphorylation (Fig. 2B). Expression of EGFR alone is not sufficient to elicit a rapid aldosterone response, as shown in Fig. 5F. The pharmacological profile is similar to ERK1/2. Aldosterone acts via MR but does not require PKC (Fig. 5, G and H).

Phosphorylation of p38 kinase was not affected by aldosterone in either CHO-MR or CHO-mock cells, as summarized in Fig. 6, A–D. Sorbitol (600 mmol/liter) served as positive control (67) and was effective in both cell types. So far, our data show that aldosterone-induced phosphorylation of the MAPKs ERK1/2 and JNK1/2 depends on MR, whereas p38 kinase is not affected by aldosterone, independent of MR expression. Thus, there seems to be a specificity of rapid, MR-dependent, aldosterone signaling.

ERK1/2 Phosphorylation in Human Aortic Endothelial Cells (HAoEC) in Primary Culture

To test whether the data for ERK1/2- and JNK phosphorylation obtained in transfected CHO and HEK cells can be transferred—at least qualitatively—to differentiated human cells in primary culture, we used HAoEC. Furthermore, aldosterone-induced changes in vascular reactivity and NO synthesis have been described (46–48). Because these cells are very sensitive to the removal of media additives, they were made quiescent for only 3 h. Consequently, the baseline level of phosphorylation was higher and the extent of stimulation—also for the positive controls—was much smaller as compared with CHO or HEK cells. A 5-min exposure to 1 μmol/liter PMA or 10% fetal calf serum enhanced ERK1/2 phosphorylation to 139 $\pm$ 4% and 131 $\pm$ 5% of control, respectively ($n = 9$; $P < 0.05$ *vs.* control for both). Aldosterone enhanced ERK1/2 phosphorylation dose dependently (Fig. 6E). The effect of aldosterone was prevented by 1 μmol/liter spironolactone (108 $\pm$ 4%, $n = 9$), 100 nmol/liter AG1478 (101 $\pm$ 1%, $n = 6$) or 100 nmol/liter PP2 (94 $\pm$ 3%, $n = 6$). As shown in Fig. 6F, aldosterone also enhanced JNK phosphorylation. In summary, these pharmacological experiments indicate that the data from transfected CHO and HEK cells can be qualitatively transferred to HAoEC.

Cytosolic Ca^{2+} Homeostasis

Finally, we tested the effect of aldosterone on cytosolic Ca^{2+}, reported to be involved in rapid aldosterone signaling in many different cell types (21, 25, 68, 69). For these experiments, we used HEK cells, because due to the higher cytosol-to-plasma ratio, as compared with CHO cells, Ca^{2+} measurements are more reliable. Control HEK cells showed a rapid and reversible Ca^{2+}-response after aldosterone application (100 nmol/liter; Fig. 7, A and B), similar to what has been described for other cell types. Aldosterone (10 nmol/liter) induced similar effects. In contrast, 100 nmol/liter dexamethasone exerted virtually no effect on cytosolic Ca^{2+} ($\Delta Ca^{2+} = 10 \pm 8$ nmol/liter, $n = 120$), suggesting a specificity for aldosterone. Because the data presented in Figs. 3 and 4 show that control HEK cells do not express MR, the Ca^{2+} response is MR independent, in agreement with data from MR knockout mice (43). In order to test this hypothesis further, we transfected HEK cells with EGFP-C1-hMR. The Ca^{2+} response in HEK-EGFP-C1-hMR cells was not different from the response in control cells (Fig. 7, B and C). Finally, we subdivided transfected cells, into those expressing MR (as determined by EGFP fluorescence) and those not expressing MR and compared their Ca^{2+} responses. As shown in Fig. 7D, there was no significant difference. Furthermore, the effect of aldosterone was not reduced in the presence of 1 μmol/liter spironolactone (Fig. 7E). Mn^{2+}-quench experiments (Fig. 7, F and G) indicate that aldosterone

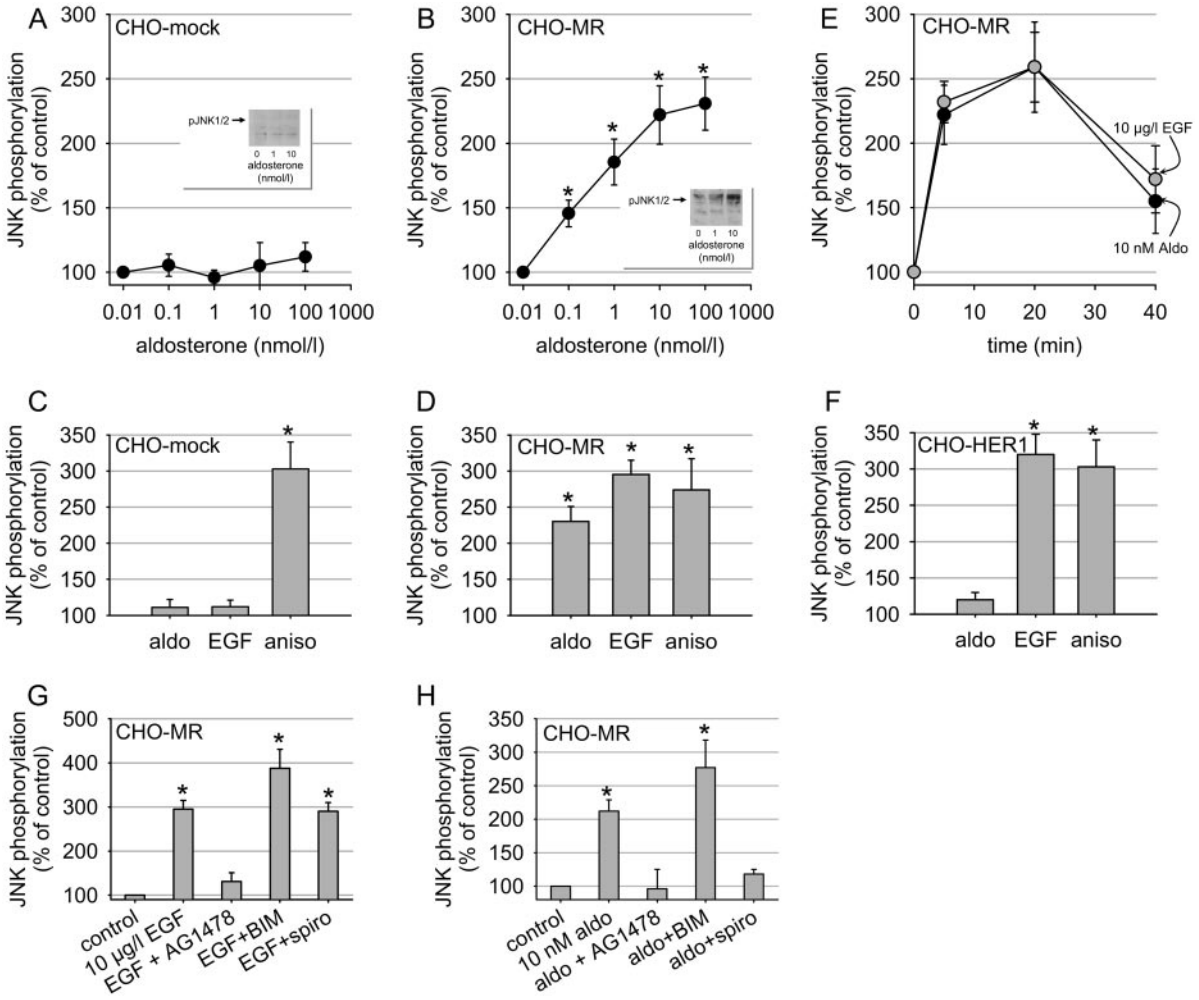

Fig. 5. Aldosterone Induces Rapid JNK1/2 Phosphorylation in CHO Cells Stably Transfected with hMR (CHO-MR)

A, A 5-min exposure of CHO-mock cells to aldosterone. B, A 5-min exposure of CHO-MR cells to aldosterone. C, A 5-min exposure of CHO-mock cells to 10 nmol/liter aldosterone, 10 μg/liter EGF, or 1 μmol/liter anisomycin. D, A 5-min exposure of CHO-MR cells to 10 nmol/liter aldosterone, 10 μg/liter EGF, or 1 μmol/liter anisomycin. E, Time course of JNK1/2 phosphorylation by aldosterone, EGF, and PMA in CHO-MR cells. F, A 5-min exposure of CHO-HER1 cells (stably transfected with hEGFR) to 10 nmol/liter aldosterone, 10 μg/liter EGF, or 1 μmol/liter anisomycin. G, EGF-induced JNK1/2 phosphorylation is inhibited by 100 nmol/liter AG1478 (EGFR kinase inhibitor) but not by 1 μmol/liter spironolactone (spiro) or 100 nmol/liter BIM (PKC inhibitor). H, Aldosterone-induced JNK1/2 phosphorylation is reduced by 100 nmol/liter AG1478 (EGFR kinase inhibitor) and by 1 μmol/liter spironolactone (spiro) but not by 100 nmol/liter BIM (PKC inhibitor). Data in the line/scatter blots were obtained by ELISA. The *insets* show representative Western blots, confirming the ELISA data. n = 9–18 for all values plotted. *, $P < 0.05$ *vs.* respective control.

stimulates Ca^{2+}-influx into HEK cells, as already shown for other cell types (21). Taken together, these data indicate that, in contrast to ERK1/2 and JNK1/2, rapid aldosterone-induced Ca^{2+} signaling does not depend on MR expression. At present, it is not known how this MR-independent rapid signaling is mediated and future studies will have to address this question in detail.

DISCUSSION

Nowadays it is clear that nongenomic effects of aldosterone—as well as of other steroid hormones—exist (22, 25). The key issues are now the physiological and/or pathophysiological relevance as well as the underlying mechanisms. This study addresses the underlying mechanisms and the data are discussed below. The physiological and pathophysiological relevance is still under intense investigation. Nongenotropic actions of aldosterone that involve the EGFR-ERK pathway can play a role in fine tuning of epithelial NaCl transport (16, 71). In principal cells of the collecting duct, EGFR activation will inhibit sodium transport, whereas in superficial enterocytes Na^+/H^+-exchange is stimulated (72). Cl^- secretion in colon crypts is also stimulated (73). Thus, aldosterone has two curbs (MR and EGFR activation) to

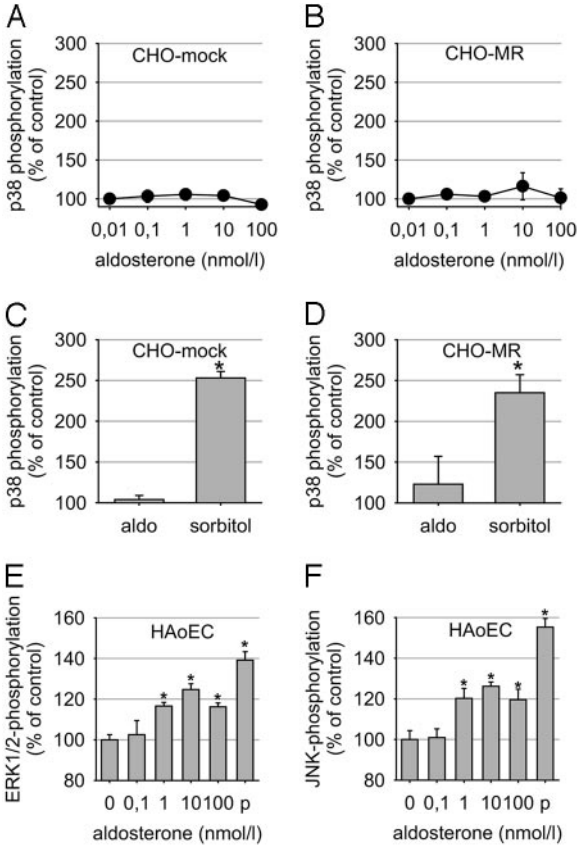

Fig. 6. Aldosterone Does Not Affect p38 Phosphorylation in CHO Cells Stably Transfected with hMR (CHO-MR)

A, A 5-min exposure of CHO-mock cells to aldosterone. B, A 5-min exposure of CHO-mock cells to 10 nmol/liter aldosterone or 600 mmol/liter sorbitol. C, A 5-min exposure of CHO-MR cells to aldosterone. D, A 5-min exposure of CHO-MR cells to 10 nmol/liter aldosterone or 600 mmol/liter sorbitol. n = 9–12 for all values plotted. *, $P < 0.05$ *vs.* respective control. E, Aldosterone affects ERK1/2 phosphorylation in HAoEC cells. P, Positive control (1 μmol/liter PMA). n = 6. F, Aldosterone affects JNK1/2 phosphorylation in HAoEC cells. P, Positive control (1 μmol/liter anisomycin). n = 6.

tune salt transport. Nongenotropic, ERK1/2-mediated actions of aldosterone in the endothelial and smooth muscle cells seem to influence vascular tone and could be important for physiological as well as pathophysiological blood pressure regulation (46–48). *In vivo* studies support the hypothesis that rapid aldosterone actions modulate blood flow by directly influencing the vasculature (74). Finally, it has been shown that the EGFR-ERK1/2 signaling pathways plays a central role in cardiovascular hypertrophy and fibrosis (75, 76). Thus, the activation of this pathway by aldosterone could contribute to its pathophysiological effects on cardiovascular tissue (7, 8).

From the data obtained in this study, we conclude that the cellular signaling network of aldosterone shows a higher degree of complexity than assumed. Of course, to what extent this complexity is cell type- and tissue-specific or general has to be investigated. In addition, one has to be aware that overexpression of signaling molecules may shift the relative importance of signaling pathways as compared with native cells.

However, as already mentioned in the introduction, there are pharmacological studies using native tissue (*e.g.* vessels) or cells with endogenous MR that emphasize the potential physiological and/or pathophysiological importance of MR-mediated rapid signaling. In addition, we show that, at least for ERK1/2 and JNK phosphorylation, the data obtained in transfected cells are qualitatively identical in human aortic endothelial cells in primary culture.

It seems that in principle we have to consider at least three tracks of cellular aldosterone signaling (Fig. 8). There is the classical, MR-dependent, genomic track, leading to the expression of so-called aldosterone-induced proteins (9–16). Next, we have a rapid, nongenotropic but MR-dependent track, leading to the activation of, at least, ERK1/2 and JNK1/2 kinase. This mechanism resembles rapid actions mediated by other steroid receptors, like ER, PR, or GR (22, 25, 33–37, 40). Although our data suggest that p38 kinase is not affected by aldosterone, indicating a specificity, we cannot exclude that other signaling modules, not investigated here, are affected. This nongenotropic, MR-dependent signaling track seems to involve cSrc and EGFR, similar to the mechanisms described for ER (24). We do not know whether such interactions are direct or mediated by additional proteins. Furthermore, the MR domains involved have to be identified in future studies, to understand the mechanism of action and the degree of specificity. It is tempting to speculate that the interaction occurs at certain domains of the plasma membrane, thereby conferring spatial-temporal specificity to the interaction. Of course, this speculation awaits further experimental evidence. Whether the signaling pathway described in the present study also involves heterotrimeric G proteins, as suggested for ER, is not known. Despite the still-missing mechanistic details, this study shows that MR can contribute to rapid signaling as already known from other steroid receptors. Like these other nonnuclear steroid receptors, the MR seems to impinge on a variety of cellular signaling pathways in various cell types (77).

Finally, there is a nongenotropic, MR-independent track, which nevertheless can distinguish between aldosterone and dexamethasone. Under our experimental conditions, this track leads to an increase of cytosolic Ca^{2+}. Comparing the pattern of the Ca^{2+}-increase with former studies (21, 25, 68, 78), it seems that aldosterone enhances Ca^{2+}-influx in HEK cells. This assumption is supported by the Mn^{2+}-quench experiments. Although not investigated here, it is conceivable that in parallel to the Ca^{2+} increase, there is also an activation of PKC, as shown in other systems (20, 25, 26, 45, 53). However, if this were true, the putative PKC activation is not necessary for the nongenotropic, MR-dependent track.

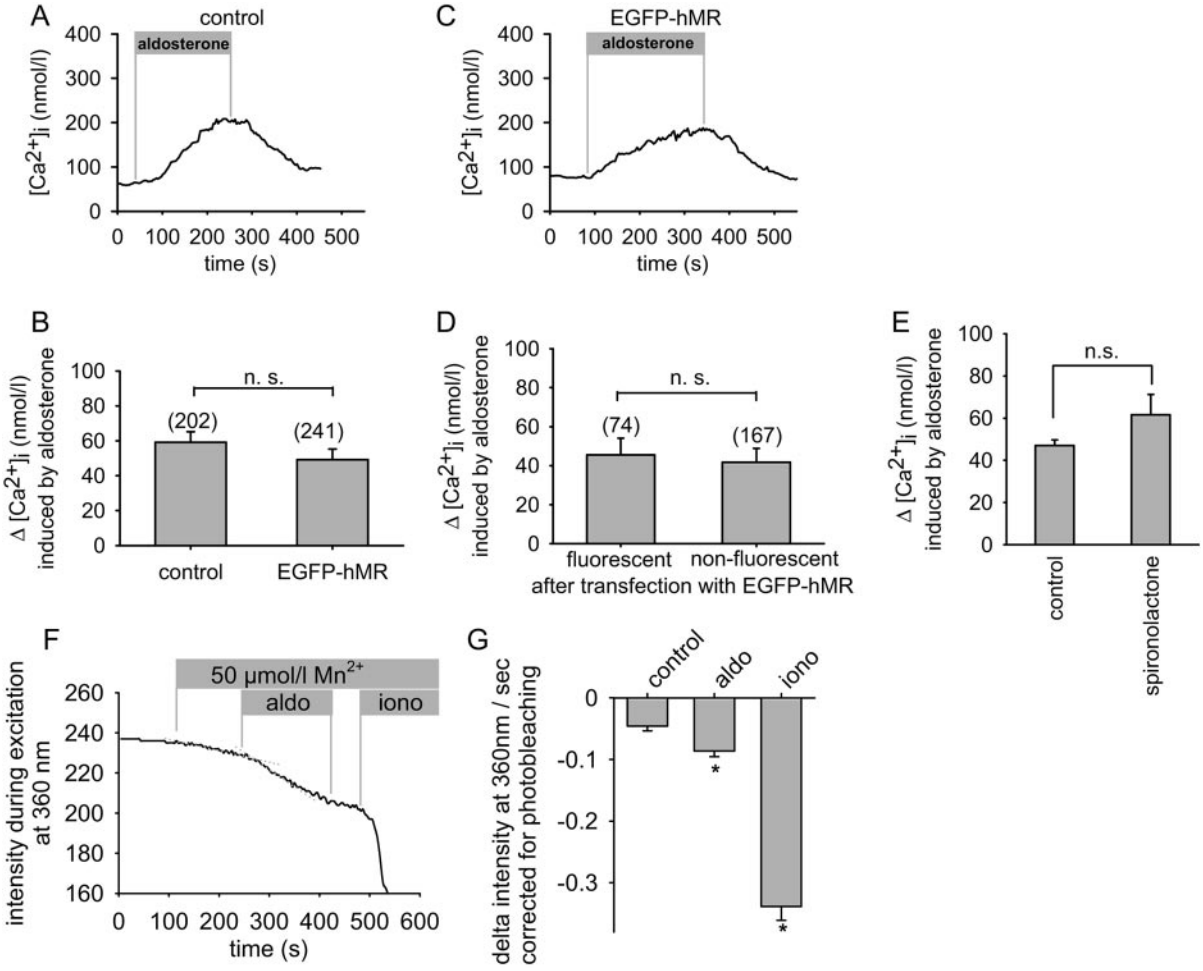

Fig. 7. Ca^{2+} Homeostasis in HEK Cells

A and C, Aldosterone induces a rapid increase of cytosolic Ca^{2+} in control cells as well as in HEK cells transiently transfected with hMR (EGFP-hMR). B, Summary of the aldosterone-induced Ca^{2+}-increase in control and EGFP-hMR-transfected cells. D, Comparison of the aldosterone-induced Ca^{2+}-increase in EGFP-hMR-transfected cells with detectable MR expression (fluorescent) and without detectable MR expression (nonfluorescent). E, Spironolactone (1 μmol/liter) did not reduced the Ca^{2+}-increase elicited by 10 nmol/liter aldosterone. n = 70. *, $P < 0.05$ *vs.* respective control. F, Aldosterone induces Ca^{2+} influx. The decrease in fluorescence (quench) in the presence of Mn^{2+} is a marker for Ca^{2+} influx. Aldosterone (10 nmol/liter) enhanced the quenching rate. The Ca^{2+}-ionophore ionomycin (1 μmol/liter) led to a dramatic increase in quenching and served as a positive control. G, Summary of the quenching rates. n = 30. *, $P < 0.05$ *vs.* control. At least four coverslips were measured for each condition.

Le Moellic *et al.* (26) reported that PKC activation supports the genomic track of aldosterone, indicating an interaction with the nongenotropic, MR-independent track. Interaction of nongenotropic and genomic events has also been reported for estrogen and progesterone (32, 56, 79, 80). Thus, not only the existence of three signaling tracks, but also the degree of their cross talk enhances the complexity of aldosterone signaling. As stated by Hammes (81) and already cited by Le Moellic *et al.* (26), the cross talk between genomic and nongenotropic actions will likely be critical for understanding the diverse biologic responses to steroids. According to the data obtained in our heterologous expression system, MR-dependent nongenotropic actions (ERK1/2 activation) can support the classical, MR-dependent, genomic track under certain circumstances. In addition to the well-established role of the genomic action of aldosterone, there is evidence for the physiological importance of the two nongenotropic signaling tracks (21, 25, 26, 29, 30, 68, 82–85). Our future challenges consist in qualitative and quantitative investigation of signaling cross talk and comparison of the relative importance of the different tracks for defined effects. Possibly the most important challenge is determination of their contribution to the pathophysiological effects of aldosterone in cardiovascular and renal tissue. In view of the RALES and EPHESUS studies (7, 8), it is necessary to understand the molecular mechanisms of aldosterone and MR action in more detail, to develop further rational therapeutic strategies.

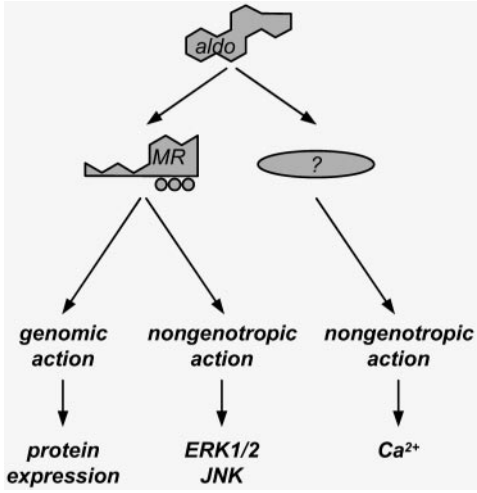

Fig. 8. Scheme of the Current Working Hypothesis of Cellular Aldosterone Signaling

There appear to exist three tracks of aldosterone signaling: 1) the classical, MR-dependent, genomic track, which leads to the expression of so-called aldosterone-induced proteins; 2) a rapid, nongenotropic but MR-dependent track, leading to the activation of ERK1/2- and JNK1/2 kinase; 3) a nongenotropic, MR-independent track, leading under our experimental conditions to an increase of cytosolic Ca^{2+}.

MATERIALS AND METHODS

Cell culture was performed as described previously (15, 50, 68). We used CHO-K1 cells and HEK-293 cells from ATCC (Manassas, VA). Twenty-four hours before the experiments, serum was removed. For the experiments presented, the cells were cultivated either on petri dishes (Becton Dickinson GmbH, Heidelberg, Germany), in 24-well plates (for reporter assay), in 96-well plates (for ELISA) or on glass coverslips (fluorescence). HAoECs in primary culture were obtained from PromoCell (Heidelberg, Germany) and cultivated according to the provided protocol using endothelial cell growth medium microvascular.

Transfection and Dilution Cloning

Transfection of the cells was performed as described before (15, 50) with the QIAGEN Polyfect reagent (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. We used the hMR expression vector pcDNA1-hMR [kindly provided by Dr. M. V. Govindan (86)], the hGR expression vector pcDNA1-hGR [kindly provided by Dr. M. V. Govindan (86)], pEGFP-C1-hMR expression vector [kindly provided by Dr. N. Farman (55)] and pEGFP-C1 (CLONTECH). Selection of transfected clones was performed with G418 (600 mg/liter).

Western Blot Analysis

Cells were lysed in ice-cold Triton X-100 lysis buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 200 μM sodium-orthovanadate, 0.1 mM phenylmethyl-sulfonyl fluoride, 1 μg/ml leupeptin, 1 μM pepstatin A, 40 mg/liter bestatin, 2 mg/liter aprotinin, 1% Triton X-100], lysis buffer according to Le Moellic *et al.* (26) (50 mmol/liter Tris-HCl, 150 mmol/liter NaCl, 1% Nonidet P-40, 2.4 mmol/liter EDTA, protease inhibitor cocktail) or radioimmunoprecipitation buffer at 4 C. Cell lysates were matched for protein content, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Subsequently, membranes were blotted with either rabbit antiphospho-ERK1/2 antibody, rabbit antiphospho-JNK1/2 antibody (1:1000, New England Biolabs, Beverly, MA), rabbit antiphospho-EGFR (no. 2234; 1:1000, New England Biolabs), rabbit antiphospho-cSrc (no. 2101; 1:1000, New England Biolabs), mouse anti-pTyr (1:1000, Santa Cruz Biotechnology, Inc.), Santa Cruz, CA), rabbit anti-GFP (1:1000, Santa Cruz Biotechnology, Inc.) or rabbit anti-EGFR (1:1000, Santa Cruz Biotechnology, Inc.). The bound primary antibody was visualized using horseradish peroxidase-conjugated secondary IgG and the ECL system (Amersham Corp., Arlington Heights, IL).

EGFP Fluorescence

Cells were cultivated on glass coverslips. Images were obtained using an inverted microscope (IM 35; Zeiss, Oberkochen, Germany) equipped with $\times$40 and $\times$100 fluorescence objectives. Fluorescence images were taken using an intensified charge-coupled device camera (Hamamatsu, Herrsching, Germany).

Quantification of ERK1/2, JNK1/2, c-Src, and p38 Phosphorylation by ELISA

For the quantification of ERK1/2, JNK1/2, and p38 phosphorylation, we performed ELISA according to Versteeg *et al.* (87) with minor modifications described previously (50). After stimulation as indicated, the cells were fixed with 4% formaldehyde in PBS and permeabilized with 0.1% Triton X-100. Cells were blocked with 10% fetal calf serum in PBS/Triton for 1 h and incubated overnight with the primary antibody (rabbit antiphospho-ERK1/2, rabbit antiphospho-JNK1/2, rabbit antiphospho-p38, rabbit antiphospho-$cSrc^{Tyr416}$; 1:1000, all from New England Biolabs). Subsequently, cells were incubated with secondary antibody (peroxidase-conjugated mouse antirabbit antibody, dilution 1:10000) in PBS/Triton with 5% BSA for 1 h at room temperature. Finally the cells were incubated with 50 μl of a solution containing 0.4 mg/ml o-phenylenediamine, 11.8 mg/ml Na_2HPO_4, 7.3 mg/ml citric acid, and 0.015% H_2O_2 for 15 min at room temperature in the dark. The resulting signal was detected at 490 nm with a multiwell multilabel counter (Victor (2), Wallac, Turku, Finland). Protein content in the wells was determined with Trypan Blue (50).

Determination of EGFP-hMR-/EGFP Expression by ELISA

EGFP- and EGFP-hMR expression was determined basically by the same method as ERK1/2 phosphorylation (see previous paragraph). HEK cells were transfected with either pEGFP-C1 or pEGFP-C1-hMR expression vector. Twenty-four hours later, cells were made quiescent and after an additional 48 h expression was determined with anti-GFP primary antibody (CLONTECH) and antimouse-horseradish peroxidase antibody. pcDNA3.1-transfected cells were used as negative controls. When MR degradation was determined, the experiments were performed in the presence of the protein synthesis inhibitor cycloheximide (10 μmol/liter) to prevent *de novo* synthesis of EGFP-hMR.

GRE Reporter Gene Assay

Transactivation was assessed by the Mercury Pathway Profiling reporter gene assay system from CLONTECH Inc. using SEAP as reporter, essentially as described earlier (88). In brief, the cells were cotransfected with pGRE-SEAP and hMR constructs or empty vectors. SEAP activity in the media was

determined with the AttoPhos System from Promega (Mannheim, Germany) and normalized to a transfection control (β-galactosidase or EGFP).

Determination of Cytosolic Ca^{2+}

Cytosolic free calcium was determined using the Ca^{2+} sensitive dye fura-2 (Molecular Probes, Leiden, The Netherlands) as described previously (68) with an inverted Axiovert 100 TV microscope (×400 magnification, oil immersion; Zeiss) and an automatic filter change device (Hamamatsu, Herrsching, Germany). The fluorescence signal was monitored at 510 nm with excitation wavelength alternating between 334 and 380 nm using a 100-W xenon lamp. The sampling rate was one ratio every 2 sec. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz *et al*. (52) using a dissociation constant of 225 nmol/liter, after subtraction of background fluorescence. Mn^{2+}-quench experiments to unveil Ca^{2+} influx were performed as described elsewhere (70). Mn^{2+} has the ability to enter the cell using Ca^{2+} channels. Once inside, the cell Mn^{2+} also bind to fura-2. If the fluorescent dye is exited at 360 nm (the wavelength where fluorescence is independent of Ca^{2+}), the intensity of the emitted light decreases as the cellular Mn^{2+} concentration increases (*i.e.* Mn^{2+} quenches the fluorescence).

Materials

U0126, tyrphostin AG 1478, tyrphostin AG112, PP2, and the protease inhibitors were obtained from Calbiochem (Bad Soden, Germany). Unless otherwise stated, all other materials were from Sigma (Munich, Germany). Control Ringer solution was composed of (mmol/liter): NaCl 130.0, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO_4 1.0, HEPES 10 and glucose 5 (pH 7.4 at 37 C), plus the respective vehicles (ethanol or dimethylsulfoxide $\leq$ 1‰).

Statistics

The data are presented as mean values $\pm$ SEM. Significance of difference was tested by paired or unpaired Student's *t* test or ANOVA as applicable. Differences were considered significant if $P < 0.05$. Cells from at least two different passages were used for each experimental series; n represents the number of cells or tissue culture dishes investigated.

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