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Colocalization of mineralocorticoid and EGF receptor at the plasma membrane

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ABSTRACT

The mineralocorticoid receptor (MR), a ligand-activated transcription factor expressed in various cell types (e.g. epithelial cells, neurons, smooth muscle cells, immune cells), plays important roles in neurohumoral, neuronal, cardiovascular, renal and intestinal function. Pathophysiological relevant signaling mechanisms include nongenomic pathways involving the EGF receptor (EGFR). We investigated whether a MR–EGFR colocalization may underlie the functional MR–EGFR interaction by coimmunoprecipitation, fluorescence resonance energy transfer (FRET) and confocal microscopy in a heterologous expression system. EGFR and a small fraction of MR colocalize at the cell membrane, independently of short time exposure (≤60 min) to receptor ligands. Twenty-four-hour-exposure to saturating concentrations of aldosterone (10 nmol/l) resulted in an almost complete nuclear translocation of MR and disappearance of MR–EGFR colocalization. EGFR transactivation is enhanced only after MR stimulation. Inhibition of HSP90 by geldanamycin did not reduce the fraction of MR interacting with EGFR. Disruption of cholesterol-rich membrane domains by cyclodextrin reduced MR–EGFR interaction. In conclusion, a subfraction of MR interacts with EGFR at the plasma membrane in our heterologous expression system, possibly at cholesterol-rich domains, to form a steroid receptor/growth factor receptor signaling module.

1. Introduction

The mineralocorticoid receptor (MR) belongs to the steroid receptor family and is usually described as a ligand-inducible transcription factor, controlling the expression of target genes involved e.g. in Na^+ and K^+ homeostasis as well as blood pressure regulation and tissue remodeling [1,2]. MR promotes pathological alterations of cardiovascular and renal tissue homeostasis, independently of its effects on blood pressure or NaCl homeostasis [3–5].

Nowadays the existence of nongenomic signaling pathways of steroid hormone receptors – including the mineralocorticoid receptor – is well accepted [6,7] and these pathways are of potentially pathological relevance [8–10]. Nongenomic signaling of MR – similar to other steroid hormone receptors – relies on a crosstalk with other cytosolic signaling cascades. In recent years, the prominent role of the EGF receptor (EGFR) in mediating at least part of nongenomic MR signaling and possibly of its pathological actions has been characterized functionally [9,11–16]. Stimulation of MR leads to a rapid EGFR transactivation [17,18]. Subsequently, cytosolic signaling cascades are activated, including extracellular regulated kinase (ERK1/2), Rho kinase or p38 kinase, which may transduce the signal into the nucleus via the transcription factors AP1 and NFkB [8,18–20], thereby eliciting an indirect genomic action. Functional data indicated that there is the possibility of a constitutive interaction of MR^{EF} with EGFR in the apparent absence of ligand [8]. Such an interaction could be explained by the colocalization of a small subset of MR with the EGFR at the plasma membrane [8]. Accordingly, Ziera et al. detected MR in the membrane protein fraction of HEK cells transfected with MR [21].

Likewise, the classical receptors for estrogens (ER), progesterone (PR), androgens (AR) and glucocorticoids (GR) also mediate at least part of their nongenomic effects [6,7,22–29] via a receptor fraction located in or at the plasma membrane (e.g. ER, [30–32]) that interacts with the EGFR. Localization of the small ER subfraction at the cell membrane depends on the E-domain and seems to occur as monomer [31].

Thus, EGFR serves as a relay station to transduce nongenomic steroid receptor signaling. However, it is not clear whether a subpopulation of steroid receptors is organized in an EGFR signaling microdomain, possibly at the plasma membrane. The functional importance of a spatial organization at the plasma membrane for rapid aldosterone/MR signaling has been suggested for pathophysiological effects in vascular smooth muscle cells [33].

In the present study, we addressed this question for the MR using immunoprecipitation, FRET and confocal microscopy. Our data contribute to further our understanding of non-genomic signaling of steroid receptors by suggesting that a small subpopulation of MR colocalizes with EGFR in a signaling microdomain at the plasma membrane. Of note, our study describes the interaction in a model with heterologous MR expression. Future studies will have to investigate the interaction of endogenously expressed MR with EGFR.

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2. Materials and methods

2.1. Cell culture

Cell culture with HEK-293 cells was performed as described previously [8].

2.2. Constructs and transfection

Transfection of the cells was performed under serum-free conditions as described before [8,19] with the Qiagen Polyfect reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Experiments were performed 48 h after transfection. We used the expression vectors pEYFP-C1-hMR ([34]), pEYFP, pcDNA3.1-hMR, pcDNA3.1-LacZ (Invitrogen). pcDNA3.1 vector contains a *Xpress* tag. Construction of the truncated versions of the hMR, which lack either the N-terminal AB-domain (hMR^{CDEF}), the AB-domain and the DNA-binding domain (hMR^{DEF}) or additionally the hinge region (hMR^{EF}) has been described previously [8]. To exclude that the tags modify the characteristics of the receptor, we compared GRE activation and nuclear translocation properties of tagged and untagged MR but could not find significant differences [19,34].

2.3. Immunoprecipitation and Western blot analysis

Cells were washed, harvested and lysed in RIPA buffer [35]. Lysates were then centrifuged at 11000 rpm at 4 °C for 10 min and the supernatant was incubated over night with EGFP or EGFR antibody (Santa Cruz Biotechnology Inc.) with end-over-end rotation and then with A/G plus agarose for another 24 h. After 10 min of centrifugation at 10000 rpm at 4 °C, the pellet was mixed with 40 µl Laemmli buffer and separated by an 8% SDS-PAGE gel.

2.3.1. Western blot analysis

Cell lysates were matched for protein content, separated by SDS-PAGE and transferred to a nitrocellulose membrane. Subsequently membranes were blotted with the respective antibodies (anti-EGFP, anti-EGFR, anti-pTyr or anti-MR from Santa Cruz; anti-Xpress from Invitrogen). The bound primary antibody was visualized using horseradish peroxidase-conjugated secondary IgG and the ECL system (Amersham Corp.). Densitometry analysis was performed with Quantity One (Biorad).

2.4. Immunofluorescence and confocal microscopy

Cells transfected with EGFP-hMR were cultivated on glass cover slips, fixed with 4% formaldehyde (15 min), washed three times with PBS and permeabilized with 0.5% Triton-X100. Subsequently, cells were incubated for 10 min in 1% SDS/PBS, followed by 10 min 100 mmol/l glycine/PBS and finally for 20 min in 10% serum/1% BSA/PBS. After 60 min incubation with anti-EGFR, the cells were washed three times with PBS and incubated for 45 min with anti-rabbit-Alexa568 secondary antibody or non-labeled secondary antibody. After three further washes, the cells were analyzed by confocal microscopy (Radiance 2000, Bio-Rad) and the images were processed using the software MetaMorph Imaging System (Microsoft).

Quantitative evaluation was performed in a blinded fashion. The observer first identified cells successfully transfected with EGFP or EGFP-MR and stained for EGFR (for an example see Fig. 4) and determined the percentage of cells showing colocalization (yellow). The results were analyzed by the Chi^2-test. Two hundred cells were evaluated.

2.5. Fluorescence resonance energy transfer (FRET)

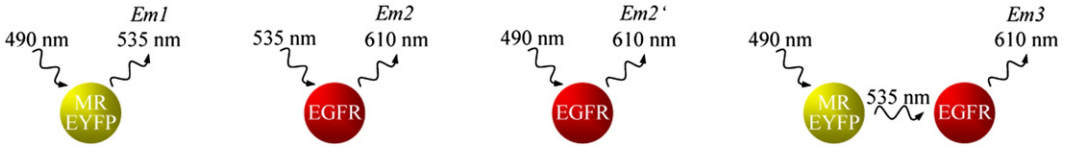
Cells were cultivated in 96-well plates and transfected with the mentioned EYFP-labeled constructs as described above. After 48 h, cells were incubated for an additional 60 min with the compounds given in the text or vehicle. Subsequently, cells were washed with PBS, fixed (4% paraformaldehyde, 60 min at 21 °C) and permeabilized (0.1% Triton X-100). After 60 min incubation in blocking solution (0.1 % Triton X-100 in TBS + 5% BSA), cells were incubated overnight with anti-EGFR (Santa Cruz; 1:100, 4 °C) or control solution. After three washes with blocking solution, cells were incubated for 60 min with anti-rabbit-Alexa568 secondary antibody (1:100). After another three washes, the following fluorescence values ($F_{excitation/emission}$) were measured using the Infinite 200 Multiwell Reader (Tecan), which supports the FRET detection mode (Fig. 1): $F_{490/535}$ (Em1 for EYFP), $F_{535/610}$ (Em2 for Alexa568, i.e. EGFR) and $F_{490/610}$ (Em3 for FRET determination). The mentioned excitation and emission wavelengths were set by two prisms with a bandpass of 9 nm for excitation and 15 nm for emission.

FRET efficiency was calculated from the fluorescence values after background subtraction as follows:

For each experiment, a set of cells not expressing the EYFP label was measured. The obtained $F_{490/610}$ value was used to determine the relative spill-over of Alexa568 emission during FRET determination (= Em2′; 20.4 ± 0.5%). Emission during FRET determination (Em3) was corrected for this spill-over (Em3′ = Em3 − Em2′). Next the fluorescence emission ratio Em3′/Em1 ($FR_{610/535}$), which is a measure for the Alexa568 (i.e. EGFR) fluorescence elicited by the EYFP emission, was calculated. In order to obtain the true $FRET_{610/535}$, the values obtained from EYFP or EYFP-MR expressing cells in the absence of anti-EGFR (resulting from EYFP spill-over) were subtracted from the values in the presence of anti-EGFR ($FRET_{610/535} = FR_{610/535}^{+Anti\text{-}EGFR} - FR_{610/535}^{-Anti\text{-}EGFR}$). Finally, we determined $FRET_{610/535}$ for EYFP and EYFP-MR under all experimental condition to obtain the MR-dependent FRET signal.

2.6. Reporter gene assay

Transactivation was assessed by the Mercury® Pathway Profiling reporter gene assay system from Clontech Inc. using secretory alkaline



Em2′ = *Em2* spill-over during *Em3* measurement

Em3′ = *Em3* - *Em2′*

Fluorescence Emission Ratio, $FR_{610/535}$ = Em3′ / *Em1*

$FRET_{610/535} = FR_{610/535}^{+Anti\text{-}EGFR} - FR_{610/535}^{-Anti\text{-}EGFR}$

MR-dependent $FRET_{610/535} = FRET_{610/535}^{EYFP\text{-}MR} - FRET_{610/535}^{EYFP}$

Fig. 1. FRET determination. Scheme showing the procedure applied to obtain an MR-dependent FRET signal. For details see Materials and methods.

phosphatase (SEAP) under the control of GRE (glucocorticoid response element) cis-regulatory response elements as reporter, essentially as described earlier [36,37]. In brief, the cells were cotransfected with pGRE-SEAP and hMR or empty vector. SEAP-activity in the media was determined with the AttoPhos® System from Promega (Mannheim, Germany) and normalized to the transfection control (beta-galactosidase).

2.7. Statistics

The data are presented as mean values ± SEM. Significance of difference was tested by paired or unpaired Student's t-test, ANOVA or Chi^2-test 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 tissue culture dishes investigated.

3. Results

3.1. Coimmunoprecipitation of MR and EGFR

As shown before [8,19] wild type HEK cells do not express detectable MR but are readily transfectable. In cells transfected with human MR, we detected EGFR in anti-MR immunoprecipitates (Fig. 2A). This was not the case in cells transfected with the empty vector. These data show that EGFR coimmunoprecipitates with MR, indicating a physical interaction of the two receptors. In our coimmunoprecipitation experiments, this interaction was not enhanced by the MR agonist aldosterone (10 nmol/l, 60 min exposure, Fig. 2A). To exclude a non-specific interaction with the EGFP-tag, we compared cell expressing EGFP-MR with cells expressing EGFP. As shown in Fig. 2B, EGFR coimmunoprecipitation occurred only when EGFP-MR was expressed, excluding an interaction with the EGFP-tag.

Next, we precipitated EGFR and determined coimmunoprecipitation using Xpress-tagged MR. To exclude non-specific interactions with the Xpress-tag, we compared cell expressing Xpress-MR with cells expressing Xpress-beta-Gal. As shown in Fig. 2C, Xpress-MR was coimmunoprecipitated with EGFR, again independent of aldosterone. We also detected traces of Xpress-beta-Gal, most probably resulting from incomplete washing. Comparison of the IP/lysate-ratio for both proteins (Fig. 2C) shows that there is a significant coimmunoprecipitation of Xpress-MR.

As already mentioned, the coimmunoprecipitation results were not affected by MR activation with aldosterone (10 nmol/l, 60 min exposure). In contrast, aldosterone enhanced EGFR tyrosine-phosphorylation in cells expressing MR, but not in controls (Fig. 2C). These data indicate that a fraction of MR colocalizes with EGFR irrespective of MR activation but that MR-induced EGFR transactivation is enhanced by aldosterone. When we exposed the cells for 24 h to aldosterone, no coimmunoprecipitation of MR and EGFR was observed (Fig. 2D). These findings indicate that the

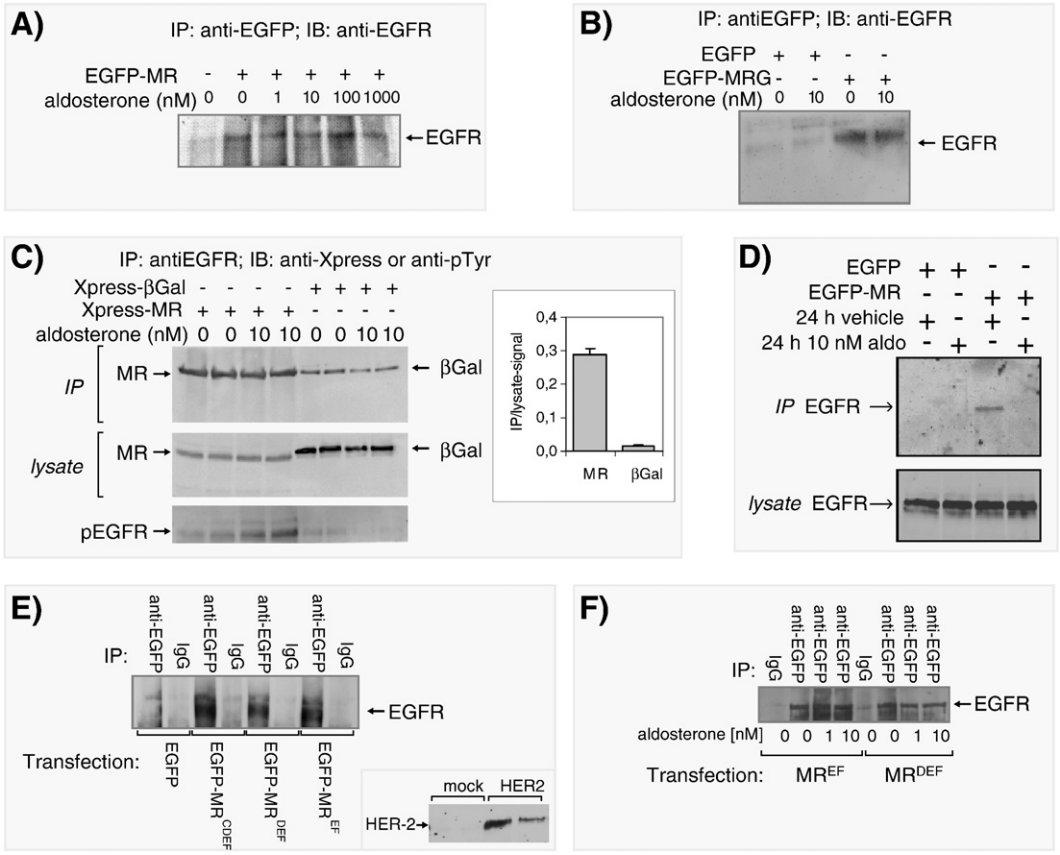


Fig. 2. Coimmunoprecipitation. (A) HEK cell transfected with EGFP-MR and wild type cells were compared. After immunoprecipitation with anti-EGFP, the EGFR was detected only in cells expressing MR, independently of aldosterone (60 min incubation). (B) HEK cell transfected with EGFP-MR or with EGFP were compared. After immunoprecipitation with anti-EGFP, the EGFR was detected only in cells expressing MR, independently of aldosterone. (C) HEK cells transfected with Xpress-MR or Xpress-beta-Gal were compared. After immunoprecipitation with anti-EGFR, the relative amount of Xpress-MR in the precipitate was significantly larger as compared to Xpress-beta-Gal. Again, the effect was independent of aldosterone (60 min exposure). pEGFR = Tyr-phosphorylated EGFR. (D) Coimmunoprecipitation of MR and EGFR was not observed in cells that had been exposed to aldosterone for 24 h, indicating that the MR is not stably associated with the cell membrane but detaches with slow kinetics after ligand binding. (E) HEK cell transfected with EGFP-MR^{CDEF}, EGFP-MR^{DEF}, EGFP-MR^{EF} or with EGFP were compared. After immunoprecipitation with anti-EGFP, the EGFR was detected in cells expressing EGFP-MR^{CDEF}, EGFP-MR^{DEF}, EGFP-MR^{EF} but not in cells expressing EGFP. The insert shows that the cells used express detectable levels of HER2 only after transfection with a HER2 construct. (F) Coimmunoprecipitation of EGFR in cells expressing EGFP-MR^{DEF} or EGFP-MR^{EF} was independent of aldosterone.

subset of MR is not stably associated with the membrane but detaches slowly and are in agreement with the previously observed disappearance of MR from the membrane fraction of HEK-MR cells after prolonged aldosterone exposure [21].

Finally, we performed coimmunoprecipitation experiments using truncated MR variants [8]. The results presented in Fig. 2E and F indicate that the MR-domains EF are sufficient for the receptor colocalization. When we used the MR-construct consisting of the domains A and B, which has previously been shown not to elicit non-genotropic actions of aldosterone, we did not observe any coimmunoprecipitation of EGFR.

In Fig. 2E, EGFR seems to present with a double band. In order to exclude that HER2 was detected incidentally, we determined HER2 expression in our cells using the HER2-antibody from Santa Cruz (sc-284). As shown in the insert in Fig. 2D, HER2 was detected only in HEK cells transfected with a HER2 construct (kindly provided by Dr. J. Troppmaier, University of Innsbruck, Austria). These data exclude the possibility of crossreactivity with HER2.

3.2. FRET of MR and EGFR

To investigate the hypothesis of MR–EGFR colocalization by a second, independent technique, we applied FRET analysis as described in the Materials and methods section. Since we used an antibody approach to label EGFR, a positive FRET value by itself would not allow the conclusion that the two receptors localize within a distance of ≤10 nm because the antibody could bridge a certain gap. Nevertheless, the conclusion of localization in close proximity is justified. Fig. 3A shows the fluorescence emission ratio $FR_{610/535}$ for cells expressing either EYFP or EYFP-MR in the absence or presence of EGFR labeling. The values obtained for EYFP with or without EGFR labeling and for EYFP-MR without EGFR labeling showed no statistical significant difference. In contrast, the value for EYFP-MR with EGFR labeling was statistically significantly higher. The differences in $FR_{610/535}$ with and without EGFR labeling yield the $FRET_{610/535}$ values, shown in Fig. 3B, which are significantly different from 0 only for EYFP-MR. MR-dependent FRET is shown in Fig. 3C. Similar to the coimmunoprecipitation results, aldosterone did not affect MR–EGFR colocalization within 60 min (Fig. 3D). In a separate set of experiments, we investigated the time-course of MR-dependent FRET in the presence of 10 nmol/l aldosterone. As shown in Fig. 3D, aldosterone induced a significant decrease of the MR-dependent FRET signal after 120 min and 24 h exposure (Fig. 3D). After 24 h exposure, the FRET signal was no longer statistically significantly different from background.

The MR antagonist spironolactone (1 μmol/l; Fig. 3D) reduced colocalization in part whereas EGF (100 μg/l; Fig. 3D) exerted no significant effect.

Inhibition of the chaperone HSP90 with geldanamycin (2 μmol/l), which is supposed to result in HSP90-MR dissociation, did not reduce the FRET signal (Fig. 3D). In contrast, geldanamycin abrogated aldosterone-induced MR transactivation activity (6.1±0.2-fold in the absence and 0.9±0.1-fold in the presence of geldanamycin, N=12). From these data, we conclude that HSP90 is not required for the colocalization of MR and EGFR. MR-dependent $FRET_{610/535}$ values significantly different from 0 were also obtained for the MR^{EF} construct (0.012±0.003, $N=5$), confirming the coimmunoprecipitation results (Fig. 2F).

Cyclodextrin (10 mg/l), which depletes the plasma membrane of cholesterol [33] reduced the MR-dependent $FRET_{610/535}$ values significantly (Fig. 3D). Although the final proof is still missing, these data suggest that EGFR-MR interaction occurs at cholesterol-rich domains (caveolae, lipid rafts).

3.3. MR and EGFR colocalize at the cell membrane

Finally, we investigated the cellular site of MR–EGFR colocalization by confocal microscopy. Fig. 4A and B show two examples of cells transfected with EGFP-MR (green fluorescence). EGFR is shown in red (anti-rabbit-Alexa568 secondary antibody) and colocalization in yellow (overlay images show all three colors, in the colocalization images the red and green were set to zero). In the overlay and colocalization images of Fig. 4A, a narrow yellow zone is detectable at the membranes of adjacent cells. The upper left cell in Fig. 4B, which has no direct neighbor, shows colocalization at the cell membrane along the entire circumference. From these images, we conclude that colocalization of MR and EGFR takes place mainly at the cell membrane. Fig. 4C shows cells

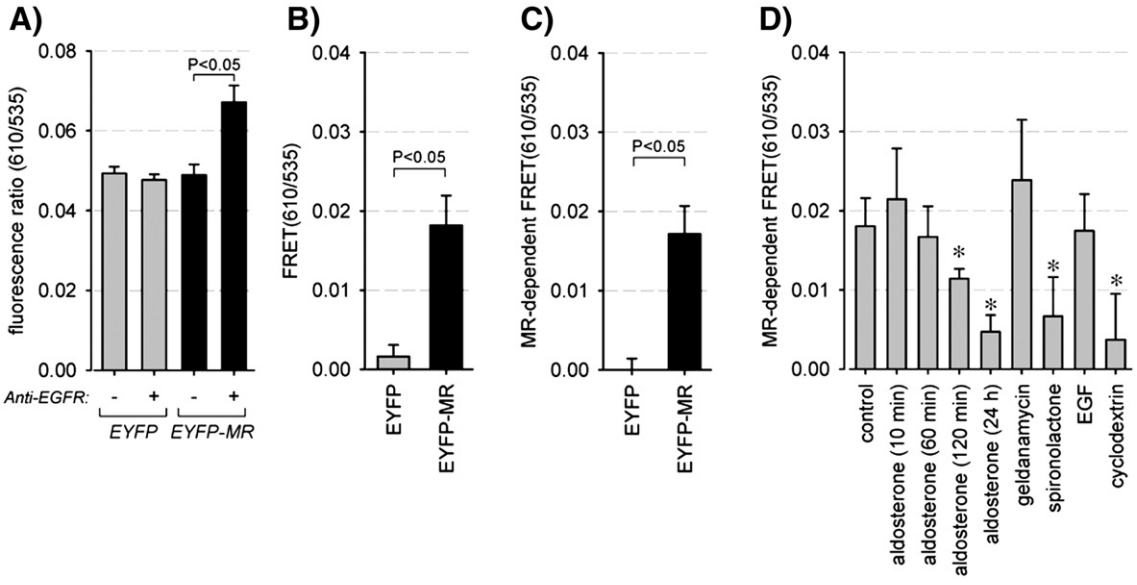


Fig. 3. FRET results. (A) Fluorescence emission ratio $FR_{610/535}$ for cells expressing either EYFP or EYFP-MR in the absence or presence of EGFR labeling (with anti-EGFR). $N=15–30$. (B) The differences in $FR_{610/535}$ with and without EGFR labeling, yielding the $FRET_{610/535}$ values are shown. (C) MR-dependent $FRET_{610/535}$ values, obtained after subtraction of $FRET_{610/535}$ values for EYFP alone, are shown. (D) Effect of aldosterone (10 nmol/l), geldanamycin (2 μmol/l), spironolactone (1 μmol/l), EGF (100 μg/l) and cyclodextrin (10 mg/l) on MR-dependent $FRET_{610/535}$ values. $N=9–25$. $^{*}=p<0.05$ versus control (=vehicle). Cells were incubated for 60 min if not stated otherwise.

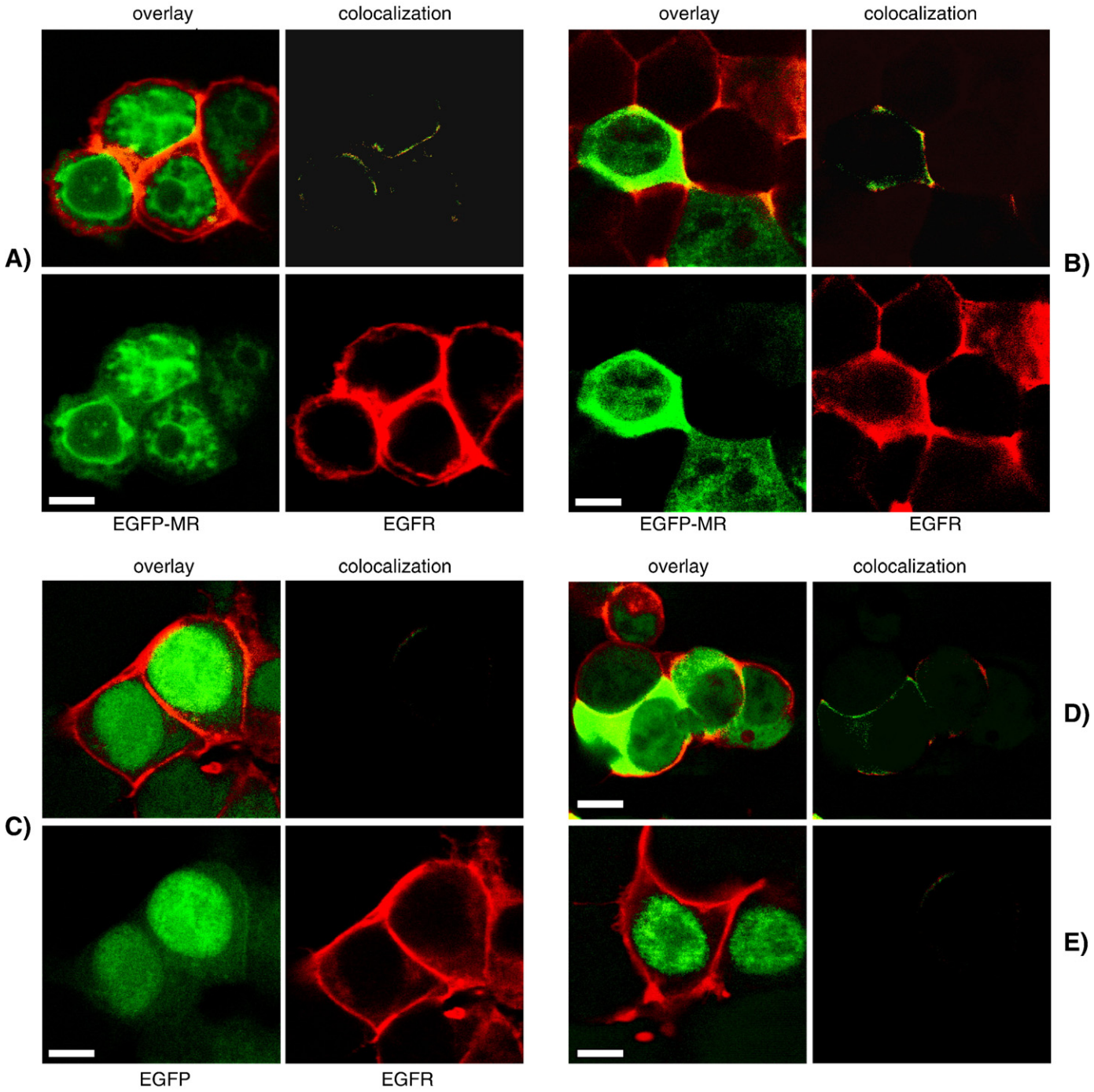


Fig. 4. Confocal microscopy. (A, B) Two examples of cells transfected with EGFP-MR (green fluorescence). EGFR is shown in red (anti-rabbit-Alexa568 secondary antibody) and colocalization in yellow (overlay images show all three colors, in the colocalization images the red and green were set to zero). (C) Example of cells transfected with EGFP. (D) Colocalization of EGFR and EGFP-MR can still be observed after incubation with geldanamycin (2 μmol/l, 60 min). (E) No colocalization can be observed after 24 h of incubation with 10 nmol/l aldosterone. Scale bar = 10 μm.

transfected with EGFP. Despite the stronger green fluorescence (as compared to EGFP-MR), there is barely any overlay detectable. Finally we confirmed the conclusion that EGFR-MR interaction is not affected by HSP90 inhibition with geldanamycin, drawn from the FRET experiments. Fig. 4D shows the colocalization signal in cells treated with geldanamycin (2 μmol/l) for 60 min. These data support the FRET results shown in Fig. 3D. Fig. 3E shows, in accordance with the immunoprecipitation and FRET data, that after prolonged exposure to a saturating concentration of aldosterone (10 nmol/l, 24 h) no colocalization signal is detectable and MR has translocated to the nucleus.

Fig. 5 shows the quantitative analysis of the confocal images. The percentage of cells successfully transfected and stained for EGFR that showed colocalization (positive cells) is presented.

4. Discussion

Nowadays the existence of nongenomic effects of the mineralocorticoid receptor – as well as of other steroid hormone receptors – is well accepted [6,7,31]. However, the underlying mechanisms are not well understood. It has been shown that MR elicits its nongenomic effects, at

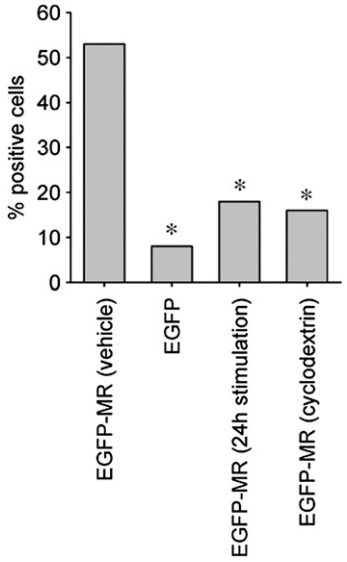


Fig. 5. Quantitative analysis of confocal images. The observer first identified cells successfully transfected with EGFP or EGFP-MR and stained for EGFR and subsequently determined the percentage of cells showing colocalization. * = $p<0.05$ versus EGFP-MR (vehicle). Two hundred cells were analyzed.

least in part, by transactivation of the EGF receptor [10,18,19,38–40]. Therefore, the MR employs a signaling cascade similar to ER and PR [6,22,29–31,41,42]. Recently, it was shown that the MR also employs the C-terminal domains EF, which comprise the ligand-binding site of the receptor, for nongenomic signaling [8] leading to EGFR transactivation at the cell membrane and thereby inducing phosphorylation of ERK1/2 [15,19,43]. In addition, a ligand-independent effect of MR on ERK1/2 activation seems to exist. These nongenomic actions seem to contribute to pathophysiological events, like enhanced collagen III abundance [8].

The data presented here extend our knowledge on the underlying mechanisms showing that there is a colocalization of a small fraction of MR with EGFR at the cell membrane. We are aware of the fact that our data were generated in a heterologous expression system and have to be extended to endogenously expressed MR and to primary cells in future. However, these approaches are hampered by the limited availability of suitable antibodies and the fact that the fraction of MR at the membrane is expected to be very small – as also shown by our results. Our data do not exclude the possibility that the colocalization is mediated by bridging/scaffold proteins, which form a larger signaling module containing MR and EGFR.

The colocalization of MR and EGFR was not dependent on receptor activation, suggesting a constitutive process. A similar ligand-independent interaction with EGFR has been described for β1-adrenergic receptors [44]. This process is different from heterodimerization with the glucocorticoid receptor or homodimerization [45], which both depend on the presence of ligands. EGFR transactivation (phosphorylation) was enhanced by the addition of aldosterone, indicating that constitutive colocalization is not sufficient for full functional interaction. Possibly, hormone binding to MR elicits conformational changes stimulating the transactivation mechanism, which may involve cSrc kinase [10,18,19,38–40]. The mechanistic integration of cSrc in the observed colocalization awaits future elucidation. Prolonged MR activation with saturating concentrations of aldosterone led, as expected, to nuclear translocation of the major fraction of the receptor. Because under this condition MR–EGFR interaction was no longer observed by any of the three techniques, we suggest that the membrane fraction of MR is not stable but dissociates slowly under conditions of maximum stimulation and also moves into the nucleus. These data are in agreement with the previously observed disappearance of MR form the membrane fraction of HEK-MR cells after prolonged aldosterone exposure [21].

MR does not contain a perfect palmitoylation sequence which has been shown to be required for membrane localization of ER, PR and AR [46]. Whether the imperfect sequence FYQLTKLL or the sequence FPAMLVEII in the E-domain is involved in MR-targeting to the membrane has to be determined in future studies. For this purpose, it has to be investigated (i) whether MR is palmitoylated, (ii) where such a palmitoylation occurs and (iii) whether it contributes to subcellular receptor localization. Since prolonged exposure to saturating concentrations of aldosterone abolished the MR–EGFR interaction, the MR membrane subset is probably not stably associated with the membrane, opening up the possibility that lipid modification of MR is not required for the observed effects. In conclusion, our data suggest the existence of signaling microdomains at the cells membrane which harbor MR and EGFR and may organize their nongenomic interaction.

Acknowledgments

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