Aldosterone interaction with epidermal growth factor receptor signaling in MDCK cells

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Gekle, Michael, Ruth Freudinger, Sigrid Mildenberger, and Stefan Silbernagl. Aldosterone interaction with epidermal growth factor receptor signaling in MDCK cells. Am J Physiol Renal Physiol 282: F669–F679, 2002. First published October 30, 2001; 10.1152/ajprenal.00159.2001. — Epidermal growth factor (EGF) regulates cell proliferation, differentiation, and ion transport by using extracellular signal-regulated kinase (ERK)1/2 as a downstream signal. Furthermore, the EGF-receptor (EGF-R) is involved in signaling by G protein-coupled receptors, growth hormone, and cytokines by means of transactivation. It has been suggested that steroids interact with peptide hormones, in part, by rapid, potentially nongenomic mechanisms. Previously, we have shown that aldosterone modulates Na\(^+\)/H\(^+\) exchange in Madin-Darby canine kidney (MDCK) cells by means of ERK1/2 in a way similar to growth factors. Here, we tested the hypothesis that aldosterone uses the EGF-R as a heterologous signal transducer in MDCK cells. Nanomolar concentrations of aldosterone induce a rapid increase in ERK1/2 phosphorylation, cellular Ca\(^{2+}\) concentration, and Na\(^+\)/H\(^+\) exchange activity similar to increases induced by EGF. Furthermore, aldosterone induced a rapid increase in EGF-R-Tyr phosphorylation, and inhibition of EGF-R kinase abolished aldosterone-induced signaling. Inhibition of ERK1/2 phosphorylation reduced the Ca\(^{2+}\) response, whereas prevention of Ca\(^{2+}\) influx did not abolish ERK1/2 phosphorylation. Our data show that aldosterone uses the EGF-R-ERK1/2 signaling cascade to elicit its rapid effects in MDCK cells.

epidermal growth factor; aldosterone; extracellular signal-regulated kinase 1/2; calcium; Madin-Darby canine kidney cells

THE CLASSIC GENOMIC MECHANISM of steroid hormone action involves binding to intracellular receptors, transcription, and protein synthesis. However, aldosterone can also induce rapid responses by interfering with mechanisms of regulation of intracellular pH or Ca\(^{2+}\) (6, 9, 12, 30, 43, 50), intracellular generation of inositol 1,4,5-trisphosphate (IP\(_3\)) (5), protein kinase C, and extracellular signal-regulated kinase (ERK)1/2 phosphorylation (9, 11, 26, 37). Former studies also revealed that aldosterone acts within several minutes on plasma membrane K\(^+\) conductance of different cells (29, 30, 41). These rapid actions of aldosterone are thought to be mediated by a plasma membrane receptor (47), although this putative receptor has not been identified. Recently, it has been shown that steroid hormones are able to interact with peptide hormone signaling (35, 46). For example, the interaction of progesterone with oxytocin signaling (17), as well as the interaction of estradiol with growth factor and angiotensin II signaling (28), has been described. In the case of aldosterone, an interaction with angiotensin II and vasopressin has been suggested (35, 48). Some of these effects have been shown to be incompatible with the classic genomic pathway. Rapid, potentially nongenomic, actions of steroids have recently been investigated more extensively, and there are several reports supporting the existence of such actions. The precise underlying mechanism and the physiological or pathophysiological significance are not yet understood. The interaction of steroids with peptide hormone signaling represents one possible mechanism for rapid steroid action while offering an explanation for the significance of these effects, i.e., modulation of peptide hormone signaling.

In the present study, we investigated the possible interaction of aldosterone with peptide hormone signaling in a cell line that has previously been shown to respond in a rapid, nongenomic way to aldosterone, Madin-Darby canine kidney (MDCK)-C11 cells (12). We determined the possible interaction with epidermal growth factor (EGF) receptor (EGF-R). The EGF-R has been shown to be involved in signaling events elicited by, for example, G protein-coupled receptors, growth hormone, and cytokines by means of a mechanism called transactivation (19, 27). Therefore, the EGF-R can serve as a central transducer of heterologous signaling systems. Moreover, a transcription-independent interaction of glucocorticoids with EGF-R has been reported (7). Furthermore, we have shown that aldosterone increases H\(^+\) affinity of Na\(^+\)/H\(^+\) exchange in MDCK-C11 cells by means of ERK1/2, which is a behavior similar to that of growth factors (11, 44). Thus it is conceivable that the EGF-R represents a pathway involved in rapid aldosterone signaling similar to, for example, G protein-coupled receptors.
EGF regulates cell proliferation, differentiation, and tissue repair and, at least in part, uses mitogen-activated protein kinases as downstream signals. In addition, enhanced EGF signaling has been observed in several tumor cells (19, 27). Furthermore, it has been shown that EGF does affect epithelial salt transport in a cell-specific manner, leading to either enhanced or reduced salt reabsorption (8, 22, 23, 31, 45). Our results show that aldosterone uses the EGF-R-ERK1/2 signaling pathway to elicit its rapid effects on ERK1/2 phosphorylation, Ca\(^{2+}\) homeostasis, and pH homeostasis in MDCK cells. Possibly, the EGF-R represents a membrane target for rapid effects of aldosterone.

**METHODS**

**Cell culture.** We used a subtype of MDCK cells, denominated C11 (MDCK-C11), which has been cloned recently in our laboratory (14). Cells were cultivated, as described previously (12, 14, 39), in MEM supplemented with 10% fetal calf serum at 37°C and 5% CO\(_2\). Serum was removed from the cells 24 h before the experiment. For the experiments presented, the cells were cultured either on permeable supports (Becton Dickinson, Heidelberg, Germany) in 96-well plates [for phosphorylated ERK (pERK1/2-ELISA) or on poly-l-lysine-coated glass coverslips (for Ca\(^{2+}\) and pH measurements). Because the effects were not statistically different for the three conditions, the data could be compared directly and were pooled.

**Western blot analysis.** Cells were washed three times with ice-cold PBS and lysed in ice-cold Triton X-100 lysis buffer (50 mM Tris–HCl at pH 7.5, 100 mM NaCl, 5 mM EDTA, 200 μM sodium-orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 40 μg/ml bestatin, 2 μg/ml aprotonin, and 1% Triton X-100) for 25 min at 4°C. Insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C. Cell lysates were matched for protein, separated on SDS-PAGE, and transferred to a polyvinylidene difluoride microporous membrane. Membranes were subsequently blotted with a rabbit anti-phospho-ERK1/2 antibody (New England Biolabs, Beverly, MA) or with anti-phospho-Tyr-antibody (PY99; Santa Cruz Biotechnology, Santa Cruz, CA). Anti-phospho-ERK1/2 antibody only detects ERK1 and ERK2 when catalytically activated by phosphorylation at Thr202 and Tyr204. The primary antibody was detected by horseradish peroxidase-conjugated secondary IgG visualization of the Amersham enhanced chemiluminescence system. Linearity of the signal has been verified by serial dilution, as recommended by the manufacturer. Densitometric analysis was performed by using SigmaGel 1.05 (Jandel, Corte Madera, CA).

**Quantification of ERK1/2 phosphorylation by ELISA.** For the quantification of ERK1/2 phosphorylation, we performed pERK1/2-ELISA according to Versteeg et al. (42). In control experiments, we compared the effect of EGF determined by Western blot and pERK1/2-ELISA and found no significant difference. Thus the results obtained by Western blot and pERK1/2-ELISA were pooled. Cells were seeded in 96-well plates (Maxisorp; Nunc) and serum starved for 24 h before the experiment. After stimulation, the cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature and washed three times with PBS containing 0.1% Triton X-100. Endogenous peroxidase was quenched with 0.6% H\(_2\)O\(_2\) in PBS/Triton X-100 for 20 min. Cells were washed three times in PBS/Triton X-100, blocked with 10% fetal calf serum in PBS/Triton X-100 for 1 h, and incubated overnight with the above described primary antibody (see Western blot analysis; 1:500 in PBS/Triton X-100 containing 5% BSA at 4°C. The next day, cells were washed three times with PBS/Triton X-100 for 5 min and incubated with secondary antibody (peroxidase-conjugated mouse anti-rabbit antibody, dilution 1:1,000) in PBS/Triton X-100 with 5% BSA for 1 h at room temperature and were washed three times with PBS/Triton X-100 for 5 min and twice with PBS. Subsequently, the cells were incubated with 50 μl of a solution containing 0.4 mg/ml O-phenylenediamine, 11.8 mg/ml Na\(_2\)HPO\(_4\), 7.3 mg/ml citric acid, and 0.015% H\(_2\)O\(_2\) for 15 min at room temperature in the dark. The resulting signal was detected at 490 nm with a multiwell, multilabel counter (Victor; Wallac, Turku, Finland). After the peroxidase reaction, the cells were washed twice with PBS/Triton X-100 and twice with demineralized water. After the wells were dried for 5 min, 100 μl of trypsin blue solution (0.2% in PBS) were added for 5 min at room temperature. Subsequently, the cells were washed four times with demineralized water, and 100 μl of 1% SDS solution were added and incubated on a shaker for 1 h at room temperature. Finally, the absorbance was measured at 555 nm with the ELISA reader.

**Immunoprecipitation.** Immunoprecipitation was performed as described recently (34). Briefly, cell lysates were precleared with protein A/G-Sepharose for 20 min at 4°C. To precipitate the EGF-R, anti-EGF-R antibody (clone Ab-5; Calbiochem-Novabiochem; Ref. 36; 10 μg/ml protein) was added for 2 h, followed by overnight incubation with protein A/G-Sepharose. Immune complexes were collected by centrifugation, washed three times with lysis buffer, and subjected to SDS/8%-PAGE, and phosphorylated EGF-R was detected by using anti-phospho-Tyr antibody (PY99). Densitometric analysis was performed by using SigmaGel 1.05.

**Determination of cytosolic \(\text{Ca}^{2+}\)\textsuperscript{−}.** Cytosolic free \(\text{Ca}^{2+}\) was determined by using the \(\text{Ca}^{2+}\)-sensitive dye fura 2 (5 μmol/l; Molecular Probes, Leiden, The Netherlands) as described previously (12) and by using an inverted Axiovert 100 TV microscope (×400 magnification, oil immersion; Zeiss, Oberkochen, Germany) and an automatic filter change device (Hamamatsu, Herrsching, Germany). In brief, after serum depletion for 24 h, cells were incubated with HEPES-Ringer containing fura 2 acetoxymethyl ester in a final concentration of 5 μmol/l for 15 min. Subsequently, the coverslips were mounted on the microscope stage. The fluorescence signal was monitored at 510 nm, with the excitation wavelength alternating between 334 and 380 nm, by using a 100-W xenon lamp. The sampling rate was one ratio every 2 s. Cytosolic \(\text{Ca}^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)) was calculated according to Grynkiewicz et al. (18) by using a dissociation constant \(K_d\) of 225 nmol/l, after subtraction of background fluorescence. The maximum and minimum ratios \(R_{\text{max}}\) and \(R_{\text{min}}\) were measured after addition of calibration solutions, which contained 1 μmol/l ionomycin and 1 mmol/l \(\text{Ca}^{2+}\) to determine \(R_{\text{max}}\) or 1 mmol/l EGTA and no \(\text{Ca}^{2+}\) to determine \(R_{\text{min}}\). Possible artifacts were excluded by measurement of autofluorescence of the different substances without fura 2.

**Determination of cytosolic pH.** Intracellular pH of single cells was determined by using the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (2 μmol/l; Molecular Probes) as described previously (12) and with the setup described in Determination of cytosolic \(\text{Ca}^{2+}\). In brief, cells were each incubated with MEM containing 2 μmol/l 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester for 5 min. Then, the coverslips were rinsed four times with superfusion solution to remove the dye-containing medium. The coverslips were transferred to the stage of an inverted Axiovert 100 TV microscope (Zeiss) and allowed to
equilibrate for 15 min. The excitation light source was a 100-W mercury lamp. The excitation wavelengths were 450 nm/490 nm. The emitted light was filtered through a bandpass filter (515–565 nm). The data-acquisition rate was one fluorescence intensity ratio every 2 s. After background subtraction, fluorescence intensity ratios were calculated. pH calibration was performed after each experiment by the nigericin (Sigma, St. Louis, MO) technique (39, 49), by using at least three calibration solutions in the range from pH 6.8 to 7.8. The calibration solutions contained 115 mmol/l KCl and 30 mmol/l NaCl.

**IP₃ formation.** IP₃ formation was determined by anion exchange columns, as described previously (32). In brief, cells seeded in six-well plates were preincubated during 24 h in media containing 0.5 μCi/ml [³H]inositol. Before experimentation, radioactive medium is aspirated, and the cells are washed with 3 × 2 ml HEPES-Ringer and then incubated for 30 min in 2 ml HEPES-Ringer containing 15 mM LiCl (pH 7.4). This medium was replaced with 1 ml HEPES-Ringer+15 mM LiCl, and after 10 min of incubation at 37°C, 1-mL aliquots of the Ringer Ringer+15 mM LiCl with the desired agonists were added. After 15 min of incubation, cells were lysed with 1 ml of 4 mM EDTA/1% SDS (90°C), and lysates were applied to ion exchange columns prepared as follows. Dowex-AG 1X-8 (0.5 g, formate form) was laid into 5-ml columns, washed with 2-ml aliquots of H₂O and 5 mM disodium tetraborate/60 mM sodium formate, and then inositol 4-monophosphate (IP₁), inositol 1,4-bisphosphate (IP₂), and IP₃ were eluted by subsequent addition of 2 ml of 0.1 M formic acid/0.2 M ammonium formate (IP₁), 0.1 M formic acid/0.4 M ammonium formate (IP₂), and 0.1 M formic acid/1.0 M ammonium formate (IP₃). Eluted IP₁–IP₃ were collected into scintillation vials, mixed with 10 ml of scintillation cocktail, and counted.

**Arachidonic acid release.** Arachidonic acid release was performed as described elsewhere (4; 24). In brief, cells were labeled for 24 h with 0.5 μCi of [³H]arachidonic acid (ARC, Munich, Germany). Ethylisopropyl amiloride was kindly provided by Dr. H. J. Lang from Aventis (Frankfurt, Germany). Control Ringer solution was composed of (in mmol/l) NaCl 300, KCl 5.4, CaCl₂ 1.0, MgCl₂ 1.0, NaH₂PO₄ 10, HEPES, and 5 glucose (pH 7.4 at 37°C), plus the respective vehicles (ethanol or DMSO ≤ 1%).

**Statistics.** Values are means ± SE. 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 dish investigated. For pH determinations, at least five coverslips were investigated for each experimental condition.

**RESULTS**

**ERK1/2 phosphorylation.** As shown in Fig. 1, A and C, 10 nmol/l aldosterone and 10 μg/l EGF stimulate ERK1/2 phosphorylation in MDCK-C11 cells, as described by us previously for aldosterone (11). The time of exposure was 10 min, because in a previous study it was shown that there was no difference between 5- or 10-min exposure (11). Control solutions always contained the appropriate amount of vehicle (ethanol or DMSO ≤ 1:1,000). To determine whether the side of aldosterone application, apical or basolateral, might be important, we compared the effect of aldosterone on filter-grown cells. As shown in Fig. 1A, there was only a slight difference for aldosterone but a marked difference for EGF. At present, we do not know whether these data mean that aldosterone acts within the cells or just rapidly crosses the monolayer. The effects of aldosterone and EGF in cells grown to ~80% confluence on solid supports were compared with the effects in cells grown on permeable supports. We did not observe significant differences. Therefore, most of the experiments were performed with cells cultivated on solid supports, and the data were pooled with those from cells grown on permeable supports. The reason for the effectiveness of EGF in cells grown to ~80% confluence on solid supports is most probably the lack of a complete apical-to-basolateral differentiation, as observed previously (11).

We tested whether inhibition of the EGF-R-kinase by compound 56 [c56; 100 nmol/l 4-(3-bromoanilino)-6,7-dimethoxyquinazoline] (3) or tyrphostin AG1478 (100 nmol/l) reduced the aldosterone- and EGF-induced phosphorylation of ERK1/2. As shown in Fig. 1, B and C, this was indeed the case, indicating that both substances use the EGF-R for signaling. Furthermore, the data in Fig. 1, B and C, show that there is an autocrine activation loop of the EGF-R signaling cascade in MDCK-C11 cells, as already shown, because EGF-R-kinase inhibition was effective in controls (36). Thus the cells are already in a certain state of preactivation; therefore, the observed effects are smaller compared with cells that are completely silent. By contrast to aldosterone, hydrocortisone (10 nmol/l) did not affect ERK1/2-phosphorylation (Fig. 1D). These data again show that the vehicle (ethanol) in which the steroids were dissolved is not responsible for the observed effects.

**Cytosolic Ca²⁺.** Mitogenic factors, such as EGF, are known to affect cytosolic Ca²⁺ homeostasis because of an increased Ca²⁺ entry across the plasma membrane or because of the release of Ca²⁺ from intracellular stores (25, 40). Under control conditions, [Ca²⁺], was 91 ± 20 nmol/l (n = 200). Figure 2 shows that 10 nmol/l aldosterone or 10 μg/l EGF induced a small but comparable increase in [Ca²⁺], corresponding to ~100

F671

AJP-Renal Physiol • VOL 282 • APRIL 2002 • www.ajprenal.org
nmol/l. These aldosterone-induced changes in [Ca\(^{2+}\)]\(_{i}\) (Δ[Ca\(^{2+}\)]\(_{i}\)) are in agreement with previously reported changes for MDCK-C11 or skin cells (12, 21); however, they are smaller compared with changes observed, for example, in M-1 cortical collecting duct cells (20). Although the changes in MDCK-C11 cells are small, it has been shown that they contribute to Na\(^{+}/\)H\(^{+}\)-exchange activation, for example (12). Similar to the effects on ERK1/2 phosphorylation, inhibition of EGF-R kinase with compound 56 (100 nmol/l) significantly reduces the Ca\(^{2+}\) changes; n = 60–120 for each bar. *P < 0.05 vs. aldosterone or EGF, respectively.

Fig. 2. Aldosterone- and EGF-induced Ca\(^{2+}\) signaling. A: EGF (10 μg/l) or aldosterone alone (10 nmol/l) exerts a slight increase in baseline cytosolic Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_{i}\). B: summary of the Ca\(^{2+}\) changes elicited by aldosterone or EGF. The inhibitor of EGF-R kinase, c56 (100 nmol/l), reduces the Ca\(^{2+}\) changes significantly; n = 60–120 for each bar. *P < 0.05 vs. aldosterone or EGF, respectively.
cantly reduced the $\Delta$[Ca$^{2+}$], induced by aldosterone or EGF. Thus aldosterone and EGF increase Ca$^{2+}$ by means of EGF-R signaling. To rule out the possibility that the EGF-R inhibitors, although used at nanomolar concentrations, reduced signaling through toxic effects, we determined whether these substances affected the bradykinin-induced signal. However, both substances did not affect the Ca$^{2+}$ rise induced by 100 nmol/l bradykinin, which induced a Ca$^{2+}$ spike of $\sim$1,100 nmol/l. These data indicate that the EGF-R inhibitors did not act by means of toxic impairment of signaling.

**Aldosterone action depends on EGF-R-kinase activity.** To determine whether Tyr phosphorylation of the EGF-R is affected by aldosterone and EGF, we performed EGF-R immunoprecipitation (stimulation time = 10 min for all experiments). As shown in Fig. 3, A and B, aldosterone and EGF enhanced Tyr phosphorylation of the EGF-R. Figure 3A again shows that there is a certain degree of autocrine activation of the EGF-R in MDCK cells, most probably involving transforming growth factor (TGF)-$\alpha$ (36). When EGF and aldosterone were added together, the extent of EGF-R-phosphorylation was enhanced. To determine whether this enhancement also affected downstream signaling, we determined the effects of aldosterone+EGF on ERK1/2 phosphorylation and Ca$^{2+}$.

**Interaction of aldosterone and EGF.** In addition to its own effect on ERK1/2 phosphorylation, aldosterone increased the effect of 10 $\mu$g/l EGF on ERK1/2 phosphorylation (stimulation time = 10 min for all experiments). Because ERK1/2 phosphorylation seems to be the signal upstream of all other events investigated in this study, except EGF-R phosphorylation, and can be quantitated reliably by ELISA (see METHODS), we determined the ERK1/2 phosphorylation dose-response curve for EGF and EGF+aldosterone. As shown in Fig. 4A, there was a shift to the left of the dose-response curve (factor $\sim$5–10) when aldosterone and EGF were added simultaneously, compared with EGF alone. ERK1/2 phosphorylation under these conditions was again prevented by EGF-R kinase blockade, as shown in Fig. 4B.

When EGF (10 $\mu$g/l) was added in the presence of aldosterone, $\Delta$[Ca$^{2+}$], ($\sim$400–500 nmol/l) values were significantly greater when compared with the sum of the individual effects (Fig. 4, C and D). Furthermore, we observed a Ca$^{2+}$ peak in the majority of the cells before a new plateau was reached. The order of application (i.e., aldosterone then EGF or EGF then aldosterone) had no significant effect on the observed effects. To determine whether the appearance of the Ca$^{2+}$ peak was the result of enhanced IP$_3$ formation, we determined the effect of aldosterone on IP$_3$ formation. As shown in Fig. 5A, aldosterone or EGF did not induce a substantial increase in IP$_3$ formation. ATP (100 $\mu$mol/l) was used as a positive control. An enhancement of the Ca$^{2+}$ changes was also observed with 1 nmol/l aldosterone ($\Delta$Ca$^{2+}$ for 1 nmol/l aldosterone+10 $\mu$g/l EGF = 350 ± 56 nmol/l; $n = 75$). When 100 $\mu$g/l EGF was used, the enhancement of the Ca$^{2+}$ response by aldosterone was substantially smaller compared with the response when using 10 $\mu$g/l EGF (Fig. 4D), indicating that aldosterone did not induce a change in the maximum Ca$^{2+}$ response, which is in agreement with the effects observed for ERK1/2 phosphorylation. In the presence of 10 $\mu$mol/l La$^{3+}$ or when extracellular [Ca$^{2+}$] was lowered to <5 $\mu$mol/l, the changes in [Ca$^{2+}$], were almost completely abrogated (Fig. 4D), indicating the dependence on extracellular Ca$^{2+}$. As already mentioned, all control solutions contained the respective vehicles (either ethanol or DMSO $\leq 1\%$). Thus the effects observed cannot be explained by the vehicles. Inhibition of ERK1/2 phosphorylation with PD-98059 (25 $\mu$mol/l, a concentration that prevented ERK1/2 phosphorylation; data not shown) reduced the Ca$^{2+}$ signal significantly (Fig. 5B). These data indicate that ERK1/2 phosphorylation is upstream of the Ca$^{2+}$ influx. By contrast, lowering extracellular Ca$^{2+}$ to prevent aldosterone- and EGF-induced Ca$^{2+}$ signaling did not affect ERK1/2 phosphorylation (Fig. 5C).
Effects of aldosterone and EGF on arachidonic acid release and cytosolic pH. To evaluate the potential affect of the Ca\(^{2+}\) and ERK1/2 signals on cell functions, we investigated arachidonic acid release and cytosolic pH. When EGF (10 μg/l) or aldosterone (10 nmol/l) were each added to the cells, there was only a very small increase of arachidonic acid-derived radioactivity release observable (Fig. 6A). By contrast, bradykinin (100 nmol/l) induced a threefold increase in arachidonic acid-derived radioactivity release, indicating that the experimental setup worked. Simultaneous addition of EGF and aldosterone resulted in a significantly increased release, indicating a synergistic action of the two. Inhibition of PLA\(_2\) with 25 μmol/l arachidonoyl trifluoromethyl ketone prevented the stimulation of radioactivity release (Fig. 6A). Thus the observed
release of radioactivity can be attributed to PLA₂ activity. Inhibition of ERK1/2 phosphorylation with 25 μmol/l PD-98059 also prevented stimulation of arachidonic acid release (110 ± 8% of control, n = 4), as was the case when extracellular Ca²⁺ was lowered (102 ± 10% of control, n = 4).
Previously, we have shown that aldosterone stimulates Na\(^+/\)H\(^+\) exchange in MDCK-C11 cells by means of ERK1/2 and leads to cytosolic alkalinization (11). As shown in Fig. 6B, 10 \(\mu\)g/l EGF led to an alkalinization similar to that when aldosterone (10 nmol/l) was used (pH under control conditions was 7.22 ± 0.06, \(n = 75\)). Again, aldosterone enhanced the effect of EGF. The Na\(^+/\)H\(^+\)-exchange inhibitor ethylisopropyl amiloride (10 \(\mu\)mol/l) prevented alkalinization almost completely (Fig. 6B). Moreover, no significant alkalinization could be observed in the presence of 25 \(\mu\)mol/l PD-98059 (ΔpH = 0.021 ± 0.013, \(n = 30\)) or when Ca\(^2+\) influx was prevented (ΔpH = −0.040 ± 0.011, \(n = 30\)). These data indicate that aldosterone and EGF act on Na\(^+/\)H\(^+\) exchange by means of ERK1/2 and Ca\(^2+\).

**Effects of aldosterone and EGF on cell proliferation.**

EGF acts as mitogen in many different cell types. Thus we investigated whether aldosterone modulates the proliferative action of EGF. Cells were made quiescent at ~70% confluence by 24-h serum removal and were incubated for another 48 h with EGF and/or aldosterone in serum-free media. As shown in Fig. 7A, the number of cells remained virtually constant under control conditions during the 48-h incubation period. Thus any increase in the number of cells under experimental conditions must reflect cell proliferation. Figure 7A shows the effect of EGF on the number of cells. At 10 \(\mu\)g/l, EGF exerted a slight proliferative action in MDCK-C11 cells. When added alone at 10 nmol/l, aldosterone had no significant effect. However, when aldosterone (10 nmol/l) was added together with EGF (10 \(\mu\)g/l), there was a clear potentiation of the proliferative effect. When saturating concentrations of EGF were used (100 \(\mu\)g/l), aldosterone had no further effect. Thus these data nicely mirror the responses of ERK1/2 phosphorylation and arachidonic acid release and show that aldosterone modulates the proliferative action of EGF.

**DISCUSSION**

During the last several years, several reports showed that steroid hormones, such as aldosterone, can elicit rapid (<10 min), potentially nongenomic, cellular responses. The underlying mechanism(s) for the rapid actions of aldosterone are still unknown. One hypothesis is based on the interaction of steroid hormones with peptide hormone signaling. For example, the interaction of progesterone with oxytocin signaling, as well as the interaction of estradiol or glucocorticoids with growth factor and angiotensin II signaling, has been described (17, 28). In the case of aldosterone, an interaction with angiotensin II and vasopressin has been suggested (35, 48). Previously, we have shown that aldosterone increases H\(^+\) affinity of Na\(^+/\)H\(^+\) exchange in MDCK-C11 cells by means of ERK1/2, a behavior similar to the action of growth factors such as EGF (11, 44). Transactivation of EGF-R is involved in the transmission of signals triggered by other mediators, for example, hormones acting by means of heterotrimeric G proteins (19). The EGF-R is therefore considered a transducer of heterologous signaling. Thus it is conceivable that EGF-R also plays a role in the integration of rapid steroid signaling.

The results presented here show that aldosterone acts on ERK1/2 phosphorylation, cytosolic Ca\(^2+\), and pH homeostasis in a manner very similar to the effects observed during EGF exposure. These data allow the hypothesis that aldosterone also “uses” the EGF-R as a transducer of heterologous signaling, as already shown for other hormones (19). If this hypothesis is true, then we should expect that the effects of aldosterone are reduced when the EGF-R kinase is inhibited. As shown for ERK1/2 phosphorylation and cytosolic Ca\(^2+\), this was indeed the case. When EGF-R kinase was inhibited, the effects of aldosterone were significantly smaller (cytosolic Ca\(^2+\)) or even completely abolished (ERK1/2 phosphorylation). The reason for the small remaining effect on cytosolic Ca\(^2+\) can be explained by the higher sensitivity of Ca\(^2+\) measurements compared with ERK1/2 phosphorylation. If aldosterone acts on cytosolic Ca\(^2+\) and ERK1/2 phosphorylation by means of the EGF-R kinase, we would also expect that aldosterone leads to enhanced EGF-R phosphorylation. As shown here, EGF-R phosphorylation was enhanced in

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**Fig. 7.** Cell proliferation. Cells were made quiescent by 24-h incubation in serum-free media. A: subsequent 48-h incubation in serum-free media did not significantly alter the number of cells. B: 48-h incubation with 10 \(\mu\)g/l EGF induced a slight increase in the number that was potentiated significantly by 10 nmol/l aldosterone; \(n = 9\) for each bar. (10) = 10 \(\mu\)g/l; (100) = 100 \(\mu\)g/l. *P < 0.05 vs. control.

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the presence of aldosterone. Thus the two lines of evidence presented here support the hypothesis that rapid effects of aldosterone involve the EGF-R pathway, at least in the cell system investigated here. This is the first report indicating an interaction of aldosterone with Tyr kinase receptor signaling. The possible physiological and/or pathophysiological significance of this interaction is supported by the fact that aldosterone was effective at nanomolar concentrations, although more detailed investigations have to be performed in future studies. It is conceivable that elevated circulating aldosterone concentrations (for example, in patients with liver cirrhosis) may elicit part of its effects by means of an interaction with EGF-R.

The data presented here also indicate that there is a certain degree of autocrine stimulation of the EGF-R in MDCK cells (see Fig. 1), as already described (36). This autocrine activation loop most probably results from the simultaneous expression of EGF-R and transforming growth factor (TGF)-α. Thus, even though the cells were made quiescent by serum removal, there is always a certain preactivation under control conditions. The autocrine preactivation is also responsible for the fact that the observed effects of EGF are smaller compared with other cell systems such as vascular smooth muscle cells, for example (2).

Our data show that aldosterone enhances EGF-R phosphorylation followed by phosphorylation of ERK1/2. Subsequently, Ca²⁺ entry across the plasma membrane is increased (Fig. 8). This conclusion is based on the observations that prevention of Ca²⁺ entry did not abrogate ERK1/2 phosphorylation, whereas inhibition of ERK1/2 phosphorylation significantly reduced the Ca²⁺ signal. Moreover, inhibition of EGF-R Tyr kinase significantly reduced ERK1/2 phosphorylation and the Ca²⁺ signal. Furthermore, we did not observe a substantial increase in IP₃ formation, which argues against the involvement of phospholipase C and subsequent Ca²⁺ release from IP₃-sensitive stores. Finally, ERK1/2 and Ca²⁺ do then transmit the aldosterone signal to further downstream events, as in Na⁺/ H⁺-exchange activation (11, 15).

How does the interaction of aldosterone with the EGF-R affect EGF signaling in MDCK-C11 cells? We tried to answer this question pharmacodynamically by using ERK1/2 phosphorylation as a parameter. Aldosterone and EGF could interact in a simple additive manner, leading to a shift to the left of the dose-response curve, with no change in the maximum effect when both are added simultaneously. Alternatively, aldosterone could enhance the maximum effect, either additively or in terms of a potentiation of EGF. Finally, aldosterone could shift the dose-response curve of EGF to lower concentrations, thereby sensitizing the cells for EGF. As shown in Fig. 4A, aldosterone and EGF seem not to act additively in a simple manner or with respect to maximum ERK1/2 phosphorylation. At submaximum EGF concentrations, aldosterone and EGF seem to induce an overadditive ERK1/2 phosphorylation, leading to a shift to the left of the EGF dose-response curve. Thus at submaximum concentrations of EGF, the cells seem to respond more sensitively to EGF when aldosterone is present compared with the situation where aldosterone is absent. This interpretation would also explain the enhanced EGF-induced response with respect to the Ca²⁺ signal, which is downstream of ERK1/2 phosphorylation. This, of course, is a pharmacodynamic description of the modulation of EGF effects, and future studies will have to focus on the underlying mechanisms. Previously, we have shown that rapid effects of aldosterone in MDCK-C11 cells are not prevented by the mineralocorticoid receptor antagonist spironolactone (13), which is similar to results obtained in other cell types (46). In some preliminary experiments, we tested the effect of spironolactone (1 μmol/l) on aldosterone-induced ERK1/2 phosphorylation and could not detect any inhibitory action of spironolactone (data not shown). Thus it is unlikely that the interaction of aldosterone with EGF-R depends on the mineralocorticoid receptor.

What is the nature of the interaction of aldosterone with EGF-R signaling? One possibility is a direct interaction on the level of the EGF-R. A similar mechanism for steroid-hormone and peptide-hormone cross talk has been proposed for progesterone and oxytocin (17). Another possible mechanism is the involvement of additional factors, for example, Src kinase, which could link the action of aldosterone to EGF-R phosphorylation. In this case, aldosterone would have to activate the additional factor directly or by means of an aldosterone receptor, as proposed by Wehling et al. (47).

Several years ago, it was shown that aldosterone can change the metabolism of membrane phospholipids, for example, leading to an increase in the diglyceride fraction (16). Changes in the lipid environment could also affect the activation of a membrane protein such as EGF-R (1). Finally, there exists the possibility that the interaction of aldosterone with EGF-R depends on the endogenous EGF-R ligand TGF-α, known to be ex-

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Fig. 8. Present hypothesis for the interaction of aldosterone and EGF. Aldosterone activates EGF-R activation by an unknown mechanism. Subsequently, ERK1/2 phosphorylation increases and stimulates Ca²⁺ entry. These two signals lead to, e.g., Na⁺/H⁺-exchange activation and arachidonic acid release. PLA₂, phospholipase A₂; TRK, tyrosine kinase.
pressed in MDCK cells (36). Although the time course of the aldosterone action is not in favor of a mechanism requiring the cleavage of an endogenous EGF-R ligand, it cannot be ruled out completely. Future studies will focus on the mechanism underlying the interaction of aldosterone with ERG-R signaling in more detail.

What is the cellular significance of aldosterone interaction with EGF-R signaling? To gain more information regarding the possible significance of rapid steroid effects, it is important to determine changes in cell function. Our data show that aldosterone-induced modulation of EGF-R signaling indeed affects certain cell functions. Of course there are many more aspects of cell function that could be affected and have not been determined here (for example, transepithelial ion transport) but will be subject to investigation in future studies. Aldosterone used the EGF-R signaling cascade to modulate the release of arachidonic acid and the activation of Na’/H’ exchange. Stimulation of arachidonic acid release and Na’/H’ exchange will also affect cell function and/or signaling, thereby creating a complex network.

With respect to a differentiation of the direct genomic (by means of the mineralocorticoid receptor) and primary nongenomic effects, the data reported here offer a new perspective. The interaction of aldosterone with EGF-R signaling is a primary nongenomic event that may have a secondary genomic impact, for example, by means of the nuclear factor of activated transcription and serum response element. Therefore, the primarily nongenomic responses may finally result in an alternative genomic response via pathways that do not rely on the mineralocorticoid receptor.

What is the role of interaction with EGF-R signaling in the physiological response to aldosterone? Presently, the answer to this question is not known. However, it is known that EGF-R signaling modulates transepithelial ion transport and stimulates salt reabsorption in certain cell types (8, 22). Furthermore, it is known that EGF-R signaling may exert profibrotic actions (10). Thus there are certain similarities with respect to the physiological (salt reabsorption) and the pathophysiological (fibrosis) action of aldosterone and EGF. Therefore it is conceivable that the interaction of aldosterone with EGF-R signaling may support physiological and pathophysiological responses to aldosterone. However, with respect to sodium handling in the distal nephron, the actions of EGF and aldosterone are opposed, because EGF has been reported to inhibit the epithelial sodium channel (31, 45). Thus aldosterone could limit its own stimulatory action on sodium reabsorption by means of the EGF-R signaling cascade, representing a negative feedback in this case. By contrast, with respect to intestinal sodium reabsorption, EGF and aldosterone seem to act in the same direction, i.e., stimulation of sodium absorption (23). Therefore, EGF-R signaling could support the stimulatory effect of aldosterone on intestinal sodium absorption. Of course, these hypotheses have to be verified in future studies by using EGF-R kinase inhibitors to test the importance of EGF-R signaling for aldosterone effects, for example.

In conclusion, our data show that aldosterone uses the EGF-R-ERK1/2 signaling cascade to elicit rapid effects in MDCK cells. In addition, aldosterone seems to modulate the action of EGF. Possibly, the EGF-R represents a membrane target for rapid effects of aldosterone. Of course, the data presented here do not affect the importance of genomic actions of aldosterone but do add an additional, primarily nongenomic, pathway.

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REFERENCES


15. Gomperts BD, Gr; a GTP-binding protein mediating exocytosis. 


