The epidermal growth factor (EGF) regulates cell proliferation, differentiation, and ion transport using ERK1/2 as a downstream effector. Furthermore, the EGF receptor (EGFR) is involved in signaling by G-protein-coupled receptors, growth hormone, and cytokines via transactivation. It has been suggested that steroids interact with peptide hormones. Previously, we have shown that aldosterone modulates EGF responses in Madin-Darby canine kidney cells (Gekle, M., Freudinger, E., Mildenberger, S., and Silbernagl, S. (2002) Am. J. Physiol. 282, F669–F679). Here, we tested the hypothesis that human EGFR-1 can confer alternative aldosterone responsiveness with respect to ERK1/2 phosphorylation to Chinese hamster ovary cells, which do not express EGF. Wild-type Chinese hamster ovary cells did not respond to EGF or aldosterone. After transfection of human EGFR-1, the cells responded to EGF, but not to aldosterone. However, when submaximal concentrations of EGF were used, nanomolar concentrations of aldosterone potentiated the action of EGF within minutes, resulting in a leftward shift of the EGF dose-response curve. This was not the case in mock-transfected cells. The EGFR kinase inhibitor tyrphostin AG1478 or the MEK1/2 inhibitor U0126 completely prevented the effect. Furthermore, aldosterone enhanced Tyr phosphorylation of c-Src and EGFR, and an inhibitor of cytosolic tyrosine kinases (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyriociaine) prevented the action of aldosterone. Our data show that aldosterone uses the EGF-EGFR-MEK1/2-ERK1/2 signaling cascade to elicit its alternative effects. In the presence of EGF, aldosterone leads to EGFR transactivation via cytosolic tyrosine kinases of the Src family.

The classical genomic mechanism of steroid hormone action involves binding to intracellular receptors and stimulation of transcription and protein synthesis. Yet, aldosterone can also induce rapid responses by interfering with, for example, regulation of intracellular pH or calcium (1–6), intracellular generation of inositol 1,4,5-trisphosphate (7), and protein kinase C (PKC) and ERK1/2 phosphorylation (5, 8–10). Former studies also revealed that aldosterone acts within several minutes on plasma membrane K⁺ conductance of different cells (2, 11, 12). These alternative actions of aldosterone have been suggested to be mediated by a plasma membrane receptor (13), although this putative receptor has not been identified. Recently, it has been shown that steroid hormones are capable of interacting with peptide hormone signaling (14, 15). For example, the interaction of progesterone with oxytocin signaling has been described (16), as well as the interaction of estradiol with growth factor and angiotensin II signaling (17). In the case of aldosterone, an interaction with angiotensin II and vasopressin has been suggested (15, 18).

Another attractive candidate of interaction with steroids represents the epidermal growth factor (EGF). EGF regulates cell proliferation, differentiation, and tissue repair and uses, at least in part, mitogen-activated protein kinases as downstream signals. In addition, enhanced EGF signaling has been observed in several tumor cells (19, 20). Furthermore, it has been shown that EGF affects epithelial salt transport in a cell-specific manner, leading to either enhanced or reduced salt reabsorption (21–25). The EGF receptor (EGFR) has been shown to be involved in signaling events elicited by, for example, G-protein-coupled receptors, growth hormone, and cytokines via a mechanism called transactivation (19, 20). This mechanism of transactivation involves EGF activation by intracellular signaling components. These may include c-Src, JAK2, and phosphatases (26). Thus, EGFR can serve as a central transducer of heterologous signaling systems. A transcription-independent interaction of glucocorticoids or estrogens with EGF has been reported (27, 28). Recently, we showed that aldosterone also interacts with EGF signaling (29). In Madin-Darby canine kidney (MDCK) cells, aldosterone potentiates the effects of EGF on ERK1/2 phosphorylation and Ca²⁺ homeostasis, two important components of cellular signaling networks. Furthermore, aldosterone potentiates the effect of EGF on Na⁺/H⁺ exchange activity and cell proliferation.

The precise underlying mechanisms and the physiological or pathophysiological significance of this cross-talk are not yet completely understood. The interaction of steroids with peptide
hormone signaling represents one possible mechanism for alternative steroid action and at the same time offers an explanation for the significance of these effects, i.e. modulation of peptide hormone signaling.

In this study, we investigated the interaction of aldosterone with human EGFR-1 (HER1) in a heterologous expression system using Chinese hamster ovary (CHO) cells. These cells do not express EGFR under control conditions (30) and are therefore an ideal model to investigate the importance of HER1 in the alternative aldosterone action. We used HER1-transfected CHO cells and tried to answer three questions. (i) Is HER1 transfection sufficient to elicit alternative aldosterone responses? (ii) Does this response depend on the presence of EGFR? (iii) Which signaling components are used for the crosstalk between aldosterone and HER1?

Our results show that aldosterone requires the EGF-HER1-MEK1/2-ERK1/2 signaling cascade to elicit, at least in part, its alternative effects. This activating effect is mediated by cytosolic tyrosine kinases (c-Src).

EXPERIMENTAL PROCEDURES

Cell Culture—We used CHO-K1 cells from American Type Culture Collection (Manassas, VA). Cells were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum at 37 °C and 5% CO₂. 24 h prior to the experiments, serum was removed from the medium. For the experiments presented, the cells were cultivated either on Petri dishes (BD Biosciences, Heidelberg, Germany) or in 96-well plates (for pERK1/2-ELISA).

Transfection and Dilution Cloning—Transfection of the cells was performed with the QIAGEN Polyfect reagent (QIAGEN Inc., Hilden, Germany) according to the manufacturer's instructions. We used the HER1 expression vector pRK5-HER1 (31) and pBabepuro (32), which are not expressing EGFR under control conditions (30) and are therefore used for our purposes (29). This was especially important to evaluate EGFR Tyr phosphorylation directly by Western blotting and to test the hypothesis that alternative actions of aldosterone involve EGFR, we reconstituted CHO cells, which do not express EGFR, with the receptor to obtain a defined cell system for the investigation of the role of EGFR in alternative aldosterone signaling.

We next tested the effects of short-term aldosterone treatment on ERK1/2 phosphorylation. To obtain reliable quantification, the majority of the ERK1/2 phosphorylation experiments were evaluated by an ELISA method originally described by Versteeg et al. (33) and recently adapted and evaluated for our purposes (29). This was especially important because we wanted to quantitatively investigate potential synergism between aldosterone and EGF. The immunoblot and ELISA analysis depicted in Fig. 2A show that CHO-HER1 cells did not respond to aldosterone alone. However, in the presence of submaximal EGF, aldosterone enhanced ERK1/2 phosphorylation. As shown in Fig. 2B, the responsiveness of CHO-HER1 cells to EGF could be easily confirmed by the ELISA method. Furthermore, the potentiating effect of aldosterone on
EGF action was independently observed in six CHO-HER1 clones. Therefore, we believe that the action of aldosterone is due to the expression of HER1. In the presence of aldosterone and EGF, ERK1/2 phosphorylation was increased by a factor of 1.33–1.64 compared with EGF alone. The EGF-induced increase in ERK1/2 phosphorylation (pERK1/2/aldosterone–EGF − pERK1/2/EGF) was enhanced by a factor of 1.43–2.65 by aldosterone (Fig. 2). These data show that aldosterone potentiates EGF signaling via HER1. PMA-induced ERK1/2 phosphorylation was not affected by aldosterone (see Fig. 4), indicating specificity of the observed effect.

Fig. 3A shows the time course of ERK1/2 phosphorylation in the presence of 1 μg/liter EGF or EGF + 10 nmol/liter aldosterone. The steroid exerted the strongest potentiation effect during the initial phase of ERK1/2 phosphorylation. We next analyzed the concentration dependence of EGF-induced ERK1/2 phosphorylation in CHO-HER1 cells were characterized. The data were obtained by pERK1/2-ELISA. The cells did not respond to aldosterone alone. n = 6–12 for each plotted value.

Fig. 1. EGFR expression and EGF responsiveness of CHO cells. A, wild-type CHO cells did not respond to EGF or aldosterone with respect to ERK1/2 phosphorylation. Application time was 5 min. B, wild-type CHO cells transfected with empty vector (CHO-mock) did not express EGFR, whereas CHO-HER1 cells stably expressed EGFR (the blot shows clone F3). C, CHO-HER1 cells responded to EGF (10 μg/liter) with ERK1/2 phosphorylation. Application time was 5 min. D, the time dependence and concentration dependence of EGF-induced ERK1/2 phosphorylation in CHO-HER1 cells were characterized. The data were obtained by pERK1/2-ELISA. The cells did not respond to aldosterone alone. n = 6–12 for each plotted value.

Fig. 2. Interaction of EGF and aldosterone. A, in CHO-HER1 cells, aldosterone (10 nmol/liter) potentiated the effect of EGF (1 μg/liter) on ERK1/2 phosphorylation. Application time was 5 min. However, the cells did not respond to aldosterone alone. Representative data are from clone F3. Upper panel, Western blot analysis; lower panel, pERK1/2-ELISA analysis. The cells did not respond to aldosterone alone. n = 6–12 for each plotted value. B, the interaction of aldosterone and EGF was tested in six different CHO-HER1 clones (n = 9–15). In all clones, aldosterone had a significant effect. In mock-transfected cells, no interaction could be observed. *, p < 0.05. Application time was 5 min. The table shows the fold increase, determined as described under “Results.”
time course of the EGF response. Both of these effects have the potential to alter the cell biological significance of the EGF signal, as shown by us in MDCK cells for proliferation and activation of Na+/H+ exchange in a recent study (29).

In the next step, we applied a pharmacological approach to determine signaling components involved in aldosterone-induced potentiation of EGF signaling (Fig. 4A). Inhibition of the HER1 kinase by 100 nmol/liter tyrphostin AG1478 or inhibition of the MEK1/2 kinases by 1 μmol/liter U0126 completely prevented ERK1/2 phosphorylation by either EGF or aldosterone + EGF. These data show that the observed effects were indeed mediated by the HER1-MEK1/2-ERK1/2 axis. Because transactivation of EGFR has been shown to involve the cytosolic tyrosine kinase c-Src in many cases (26), we tested the c-Src inhibitor PP2 (100 nmol/liter) (Fig. 4A). In the presence of PP2, EGF still induced ERK1/2 phosphorylation. However, aldosterone was no longer able to potentiate the effect of EGF. Thus, these data indicate that aldosterone acts via c-Src. Finally, we tried to test the involvement of PKC. Inhibition of PKC by bisindolylmaleimide (100 nmol/liter) (Fig. 4A) induced per se a potentiation of EGF-induced ERK1/2 phosphorylation. These data indicate that there exits a negative feedback loop via PKC with respect to HER1 signaling under our experimental conditions, which is already known (20). This hypothesis is further confirmed by the inhibitory action of PMA on EGF-induced ERK1/2 phosphorylation (Fig. 4A, inset). Be that as it may, inhibition of PKC by bisindolylmaleimide did not prevent the potentiating action of aldosterone with respect to EGF-induced ERK1/2 phosphorylation.

In the last step, we confirmed the hypothesis of c-Src-mediated HER1 stimulation, derived from the pharmacological data, by immunoprecipitation and Western blotting. The data in Fig. 4B show that aldosterone induced HER1 Tyr phosphorylation. Furthermore, aldosterone led to Tyr phosphorylation of c-Src (Fig. 4C), and aldosterone-induced EGFR hyperphosphorylation could be prevented by PP2 (Fig. 4D). Thus, our data support the conclusion that aldosterone potentiates EGF signaling by c-Src-mediated stimulation of HER1.

**DISCUSSION**

During the last few years, several reports have shown that steroid hormones such as aldosterone can elicit rapid, so-called alternative, cellular responses. The underlying mechanism(s) for the rapid actions of aldosterone are still not well understood. One hypothesis is based on the interaction of steroid hormones with peptide hormone signaling. For example, the interaction of progesterone with oxytocin signaling has been described (16), as well as the interaction of estradiol or glucocorticoids with growth factor and angiotensin II signaling (17). In the case of aldosterone, an interaction with angiotensin II and vasopressin has been suggested (15, 18). We have previously shown that aldosterone enhances EGF signaling with respect to ERK1/2 phosphorylation and Ca2+ homeostasis in MDCK cells (29, 35).

Transactivation of EGFR is involved in the transmission of signals triggered by other mediators such as hormones acting via heterotrimeric G-proteins (19). EGFR is therefore considered a transducer of heterologous signaling. Thus, it is conceivable that EGFR also plays a role in alternative aldosterone signaling. The questions addressed in this study were as follows. (i) Is HER1 transfection sufficient to elicit alternative aldosterone responses? (ii) Does this response depend on the
presence of EGF? (iii) Which signaling components are used for the cross-talk between aldosterone and HER1?

Our data show that expression of HER1 plus the addition of EGF were sufficient to reconstitute the alternative signaling network in CHO cells. The observation that the addition of EGF was necessary for the aldosterone effect is in agreement with its action in MDCK cells, where aldosterone stimulates an autocrine EGF activation loop (29, 35). Thus, it seems that EGF/HER1 is sufficient to reconstitute the rapid activation of ERK1/2 by aldosterone. The steroid hormone leads to a sensitization of the cells to EGF (leftward shift of the dose-response curve) without enhancing the maximal effect. Cross-talk between signaling systems and EGFR can result from receptor phosphorylation, followed by dimerization and subsequent activation of the receptor kinase (19, 26, 36). In another mechanistic concept, cross-communication results from the rapid activation of metalloproteinase and cleavage of proheparin-binding EGF-like growth factor (37). Finally, inhibition of phosphatase activity in the presence of high basal receptor kinase activity has been proposed as a mechanism for transactivation (26). Common to all these scenarios is the fact that the addition of exogenous EGF does not seem to be necessary for transactivation. This is in contrast to the action of aldosterone reported here. At present, the detailed underlying processes for the action of aldosterone are not known. Possibly, aldosterone stands for a new scenario in which there is no pure transactivation, but “transmodulation” of EGF actions. However, in cells

FIG. 4. Mechanism of aldosterone-EGF interaction. A, the potentiating effect of aldosterone (aldo) with respect to ERK1/2 phosphorylation could be prevented by inhibition of c-Src (with PP2), but not by inhibition of PKC (with bisindolylmaleimide (BIM)). On the contrary, PKC seemed to exert a negative feedback on EGFR signaling because the effect of EGF was enhanced by bisindolylmaleimide. This hypothesis is supported by the fact that stimulation of PKC with PMA (inset) reduced the effect of EGF. Inhibition of MEK1/2 with U0126 or of EGFR kinase with tyrphostin AG1478 abolished the effects of EGF and aldosterone completely. Hydrocortisone (hydro) did not enhance EGF signaling. Aldosterone did not enhance PMA-induced ERK1/2 phosphorylation, indicating a certain specificity of the aldosterone-EGF interaction (n = 6–24). *, p < 0.05. Application time was 5 min. B, aldosterone enhanced EGF-induced EGFR Tyr phosphorylation. Conditions were as follows: 1 μg/liter EGF and 10 nmol/liter aldosterone. The numbers on top of the blot indicate the percentage increases over control. Application time was 5 min. pEGFR, phosphorylated EGFR. C, aldosterone enhanced c-Src Tyr phosphorylation. Application time was 5 min. pcSrc, phosphorylated c-Src. D, in the presence of PP2 (100 nmol/liter), the effect of aldosterone on EGFR phosphorylation was no longer observed. Application time was 5 min.

FIG. 5. Hypothetical model of the aldosterone-EGF interaction. Aldosterone leads to phosphorylation and stimulation of c-Src, which then co-stimulates EGFR when EGF is present. Subsequently, the Raf-MEK-ERK cascade is activated. Finally, pERK1/2 can elicit various cellular responses, ranging from the activation of Na+/H+ exchange to gene expression and proliferation. Within this signaling network, PKC can exert a 2-fold action: inhibition of EGFR and activation of ERK1/2 phosphorylation.

with an autocrine EGF activation loop such as MDCK cells (29), the addition of aldosterone has the same effect compared with other transactivating stimuli.
The concentration ranges used here for aldosterone and EGF occur in physiological and pathophysiological situations, supporting the potential relevance of our findings. The relevance at the cellular level has been demonstrated in MDCK cells, where the aldosterone-induced transactivation of the EGFR signaling network led to enhanced Na+/H+ exchange activity and cell proliferation (29, 35). Of course, the relevance in vivo has to be demonstrated in further studies using, for example, EGFR kinase inhibitors. It is known that EGFR signaling modulates transepithelial ion transport and stimulates salt reabsorption in certain cell types (21, 22). Furthermore, it is known that EGFR signaling may exert profibrotic actions (38). Thus, there are certain similarities with respect to the physiological (salt reabsorption) and pathophysiological (fibrosis) actions of aldosterone and EGF. Therefore, it is conceivable that the interaction of aldosterone with EGFR signaling may support physiological and pathophysiological responses to aldosterone.

The question that now arises is related to the mechanism by which aldosterone interacts with the EGFR signaling network. It has been shown that aldosterone can activate PKC (5). Because PKC can lead to activation of ERK1/2, this pathway is a potential candidate for the interaction of aldosterone and EGF. On the other hand, PKC can also inactivate EGFR signaling. Our data indicate that the later is also true in our system. When PKC was inhibited, the effect of EGF on ERK1/2 phosphorylation was enhanced. In addition, when PKC was stimulated by PMA, the effect of EGF was reduced. Finally, inhibition of PKC did not prevent the action of aldosterone. Thus, PKC does not seem to be the signaling module that links aldosterone to EGFR. Another signaling pathway known to be involved in EGFR transactivation is the cytosolic tyrosine kinase c-Src pathway (26, 36, 39). Activated c-Src then phosphorylates EGFR, increasing its activity. When we inhibited c-Src, the effect of aldosterone was abolished completely, indicating that the steroid indeed acts via c-Src. This hypothesis is further supported by the fact that aldosterone induced c-Src phosphorylation and therefore its activation. From these data, we can derive the following working model (Fig. 5): aldosterone stimulates c-Src, which then co-stimulates EGFR when EGF is present. Subsequently, the Raf-MEK-ERK cascade is activated. This is of special importance considering the widespread expression of EGFR, including cell types known to express the mineralocorticoid receptor such as renal epithelial and cardiac cells. For this purpose, we will used cotransfection of CHO cells with HER1 and the mineralocorticoid receptor to examine alternative and classical aldosterone signaling. In conclusion, our data show that aldosterone requires the EGF-EGFR-MEK1/2-ERK1/2 signaling cascade to elicit its alternative effects and that the EGF-EGFR system is sufficient to reconstitute some effects observed in cells with endogenous EGFR expression.

REFERENCES

1. Winter, D. C., Schneider, M. F., O’Sullivan, G. C., Harvey, B. J., and Geibel, J. (1999) J. Membr. Biol. 170, 17–26
2. Oberleithner, H., Weigt, M., Westphale, H.-J., and Wang, W. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1464–1468
3. Villeda, S., Guerra, L., Helmle-Koh, C., and Murer, H. (1992) Pflugers Arch. Eur. J. Physiol. 422, 9–15
4. Cooper, G. J., and Hunter, M. (1994) J. Physiol. (London) 479, 423–432
5. Doolan, C. M., and Harvey, B. J. (1996) J. Biol. Chem. 271, 8763–8767
6. Gekle, M., Golenhofen, N., Oberleithner, H., and Silbernagl, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10500–10504
7. Christ, M., Eisen, C., Aktas, K., Theisen, K., and Wehling, M. (1993) J. Clin. Endocrinol. Metab. 77, 1452–1457
8. Stockrand, J. D., Spier, B. J., Worrell, R. T., Yue, A.-Baldawi, N., and Eaton, D. C. (1999) J. Biol. Chem. 274, 35449–35454
9. Manegold, J. C., Falkenstein, E., Wehling, M., and Christ, M. (1999) Cell. Mol. Biol. 45, 805–813
10. Gekle, M., Freudinger, R., Mildenberger, S., Schenk, K., Marschitz, I., and Fuchs, P. (1999) Pflugers Arch. Eur. J. Physiol. 441, 781–786
11. Oberleithner, H., Kersting, U., Silbernagl, S., Steigner, W., and Vogel, U. (1989) J. Membr. Biol. 111, 49–56
12. Urban, V., van Kerkhoff, E., Maguire, D., and Harvey, B. J. (1996) J. Physiol. (London) 491, 111–130
13. Wehling, M., Christ, M., and Theisen, K. (1992) Am. J. Physiol. 263, E974–E979
14. Wehling, M. (1997) Annu. Rev. Physiol. 59, 363–383
15. Schwab, A., and Oberleithner, H. (1995) in Genomic and Non-genomic Effects of Aldosterone (Wehling, M., ed) pp. 51–76, CRC Press Inc., Boca Raton, FL
16. Grazzini, E., Guillou, G., Muller, B., and Zigang, H. (1998) Nature 392, 509–512
17. Neugarten, J., Medve, I., Lei, J., and Silbiger, S. R. (1999) Am. J. Physiol. 277, F975–F981
18. Wehling, M., Neylon, C. B., Fullerton, M., Boksh, A., and Funder, J. W. (1995) Circ. Res. 76, 973–979
19. Hackel, P. O., Zwick, E., Preznel, N., and Ulrich, A. (1999) Curr. Opin. Cell Biol. 11, 184–189
20. Mognol, N., and Sternberg, P. W. (1999) Curr. Opin. Cell Biol. 11, 190–196
21. Keeley, S. J., Urbe, J. M., and Barrett, K. E. (1998) J. Biol. Chem. 273, 27111–27117
22. Danto, S. I., Borok, Z., Zhang, X. L., Lopez, M. Z., Patel, P., Crandall, E. D., and Lubman, R. L. (1998) Am. J. Physiol. 275, C829–C929
23. Granzina, S., Tuil, E., Furuya, H., and Asano, Y. (1999) Miner. Electrolyte Metab. 25, 191–198
24. Warden, D. H., and Stokes, J. B. (1993) Am. J. Physiol. 264, F670–F677
25. Khurana, S., Naith, S. K., Levine, S. A., Bowser, J. M., Tse, C. M., Cohen, M. E., and Donowitz, M. (1996) J. Biol. Chem. 271, 9919–9927
26. Carpenter, G. (1999) J. Cell Biol. 146, 697–702
27. Croxall, J. D., Choudhury, Q., and Flower, R. J. (1990) Br. J. Pharmacol. 100, 289–298
28. Kelly, M. J., and Levin, E. R. (2001) Trends Endocrinol. Metab. 12, 152–156
29. Gekle, M., Freudinger, R., Mildenberger, S., and Silbernagl, S. (2002) Am. J. Physiol. 282, F669–F679
30. Shi, W., Fan, H., Shum, L., and Derynck, R. (2000) J. Cell Biol. 150, 591–601
31. Redemann, N., Holzmann, B., von Ruden, T., Wagner, E. F., Schlessinger, J., and Ullrich, A. (1992) Mol. Cell. Biol. 12, 491–498
32. Morgenstern, J. P., and Land, H. (1999) Nucleic Acids Res. 18, 3587–3596
33. Versteeg, H. H., Nijhuis, E., van den Brink, G. R., Evertzen, M., Pynaert, G. N., van Deventer, S. J. H., Coffer, P. J., and Peppelenbosch, M. P. (2000) Biochem. J. 350, 717–722
34. Schramek, H., Wölfingseder, D., Pollack, V., Freudinger, R., Mildenberger, S., and Gekle, M. (1997) J. Pharmacol. Exp. Ther. 283, 1460–1468
35. Gekle, M., Freudinger, R., Mildenberger, S., and Silbernagl, S. (2002) Steroids 67, 499–504
36. Bokemeyer, D., Schmitz, U., and Kramer, H. J. (2000) Kidney Int. 58, 549–558
37. Pretzel, N., Zweck, E., Dau, R., Leen, M., Abraham, R., Wallasch, C., and Ulrich, A. (1999) Nature 402, 884–888
38. El Nahas, A. M. (1992) Kidney Int. 41, S15–S20
39. Ishida, M., Ishida, T. S., and Berke, B. C. (1998) Circ. Res. 82, 7–12
40. Alzamora, R., Michea, L., and Marusic, E. T. (2000) Hypertension 35, 1099–1104
