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EGF-Stimulated Aldosterone Secretion is Mediated by Tyrosine Phosphorylation but not by Phospholipase C in Cultured Porcine Adrenal Glomerulosa Cells

We examined the effect of EGF and angiotensin II (AII) on the formation of inositol phosphates and aldosterone secretion, and observed the role of tyrosine phosphorylation in EGF or AII-mediated aldosterone secretion. As cultured glomerulosa cells were incubated with increasing concentrations of EGF (0.01-100 ng/mL), aldosterone secretion increased and reached a plateau at EGF concentration of 10-50 ng/mL. Although EGF alone did increase aldosterone secretion in glomerulosa cells, it did not enhance AII-induced aldosterone secretion when both EGF and AII were added. EGF-induced tyrosine phosphorylation peaked at around 1 min after stimulation and at a concentration of 10-50 ng/mL. AII stimulated tyrosine phosphorylation, but the stimulatory effect was less than that observed in the presence of EGF. Although the latter induced tyrosine phosphorylation of various proteins, it failed to stimulate the formation of inositol phosphates. On the other hand, AII stimulated the production of inositol phosphates in a dose-dependent manner, with maximal stimulation at 10^{-8}M. The addition of 10 ng/mL EGF did not affect the AII-induced formation of inositol phosphates. In conclusion, EGF-stimulated aldosterone secretion might be mediated by tyrosine kinase. However, since EGF did not stimulate inositol phospholipid hydrolysis in cultured porcine adrenal glomerulosa cells, its effect does not seem to be mediated by phospholipase C.

Key Words: *Zona glomerulosa; Epidermal growth factor; Angiotensin II; Aldosterone; Tyrosine; Phosphorylation; Phospholipase C*

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INTRODUCTION

By adopting different signal transduction pathways, signaling molecules such as angiotensin II (AII), K^+ or adrenocorticotropin (ACTH) stimulate adrenal glomerulosa cells and thus induce aldosterone secretion (1). When AII binds to a cognate receptor, G proteins are activated. Then they activate phospholipase C (PLC)-β, and this in turn hydrolyzes phosphatidylinositol-4,5-bisphosphate to generate two second messenger molecules, inositol trisphosphate (IP_3) and diacyglycerol (DAG) (2). IP_3 then prompts the release of Ca^{2+} from intracellular stores and DAG activates protein kinase C (PKC); this in turn activates various transcriptional factors and enzymes, resulting in aldosterone secretion. The stimulatory effect of K^+ on aldosterone secretion is mediated by the membrane depolarization-induced opening of a voltage-dependent calcium channel. ACTH stimulates aldosterone secretion through adenylate cyclase, and this generates cAMP (3, 4). On the other hand, several molecules have been known to inhibit aldosterone secretion; this inhibitory effect is mediated by activating guanylate cyclase or inhibiting adenylate cyclase (5).

Although many signaling molecules and signal transduction mechanisms are involved in the regulation of aldosterone secretion in adrenal glomerulosa cells, the role of tyrosine phosphorylation, one of the most important pathways in signal transduction, has not yet been determined. To activate IGF-I receptor, a well-known receptor tyrosine kinase, Horiba et al. (6) stimulated adrenal glomerulosa cells with IGF-I. This induced DNA synthesis, but failed to stimulate aldosterone secretion. Natarajan et al. (7), however, showed that epidermal growth factor (EGF) stimulated aldosterone secretion in rat and human adrenal glomerulosa cells. Many hormones, growth factors or cytokines stimulate tyrosine

phosphorylation, and among these, EGF, which stimulates in vitro proliferation of many different cells, has been extensively studied. EGF induces tyrosine phosphorylation of many proteins including PLC-γ, and thus stimulates the phosphoinositide signaling pathway. Furthermore, it is known to exert both metabolic and growth promoting effects and affects steroidogenesis not only in germinal cells (8-10) but also in adrenal cells; it activates the hypothalamic-pituitary-adrenal axis (11) in humans, and stimulates in vitro and in vivo cortisol secretion in sheep (12). However, the role of EGF and tyrosine phosphorylation in aldosterone secretion is not fully understood.

The most potent physiological stimulus of aldosterone secretion is AII; its signal, as previously mentioned, is mediated by G protein and PLC-β. Furthermore, many investigators have accumulated evidence to showing that AII also stimulates tyrosine kinases in vascular smooth muscle and mesangial cells (13, 14). Although its receptor does not show tyrosine kinase activity, it recruits intracellular tyrosine kinases and thus induces tyrosine phosphorylation of intracellular proteins. On the other hand, whether AII can stimulate tyrosine phosphorylation in adrenal glomerulosa cells has not been extensively investigated. Although several researchers have insisted that AII activates tyrosine kinases and thus stimulates aldosterone secretion, they were unable to directly demonstrate the phosphorylation of tyrosine; instead they used tyrosine kinase inhibitors to block AII-induced aldosterone secretion (15, 16).

In this study, we therefore investigated whether EGF-induced tyrosine phosphorylation stimulated aldosterone secretion, and studied the role of PLC in EGF- or AII-induced aldosterone secretion. We also performed a series of experiments to observe whether cross-talk, or interaction, existed in EGF- and AII-induced signaling pathways.

MATERIALS AND METHODS

Materials

DMEM, Ham's F12 medium, transferrin, fibronectin, antibiotics-antimycotics solution, and human recombinant EGF were obtained from Gibco BRL (Gaithersburg, U.S.A.). Fetal calf serum and horse serum were purchased from Hyclone Lab Inc. (Logan, U.S.A.), antiphosphotyrosine antibodies (4G10) from Upstate Biochemical Inc. (Lake Placid, U.S.A.) and human AII from BACHEM (Switzerland). A BCA protein quantification kit was purchased from Pierce Chemical Co. (Rockford, U.S.A.), and a Mighty Small™ electrophoresis kit from Hoeffer Co. (San Francisco U.S.A.). For Western blot, an electrochemiluminescent system was purchased from Amersham Co. (Buckinghamshire, U.K.). [^{3}H]*myo*-inositol was obtained from New England Nuclear (Boston, U.S.A.), and Dowex AG-1X8 anion exchange resin (200-400 meshes, formate form), to measure the formation of inositol phosphates, from Bio-Rad (Richmond, U.S.A.). A radioimmunoassay kit for aldosterone was purchased from Diagnostic Product Co. (L.A., U.S.A.). Percoll, bovine serum albumin, collagenase and deoxyribonuclease were obtained from Sigma Chemical Co. (St. Louis, U.S.A.).

Methods

Isolation of porcine adrenal glomerulosa cells

Porcine adrenal glomerulosa cells were prepared as described previously (17) with a slight modification. In brief, porcine adrenal glands were obtained from a local slaughterhouse and transported in ice-cold 0.9% NaCl solution. The adrenals were bisected and the capsular portion was separated from the remainder of the gland by slicing the outer 0.3 mm with a microtome. These tissue slices were cut into small pieces and incubated in RPMI 1640 solution containing 1 mg/mL BSA and 20 mM HEPES (pH 7.4). Crude collagenase (2.0 mg/mL) and deoxyribonuclease (0.05 mg/mL) were added to this solution, which contained the capsular portions, and the mixture was allowed to incubate at 37℃ for 2 hours in a shaking water bath in room air. During incubation, the tissue was mechanically dispersed several times by pipetting up and down through a wide-mouthed Pasteur pipette. Dispersed cells were separated from tissue fragments by filtering through four layers of gauze. After washing with HEPES-buffered Krebs-Ringer solution (HKR) containing 135 mM NaCl, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 2.5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 5.5 mM glucose, 0.1 mg/mL BSA and 20 mM HEPES (pH 7.4), the cell suspension was loaded onto a discontinuous Percoll gradient consisting of layers containing 20, 30, and 50% Percoll isomotically diluted with concentrated RPMI 1640 solution. After centrifugation at 1,500 rpm for 25 min at 4℃, the cells separated into bands with glomerulosa cells appearing at the 30 and 50% interface. This band was collected and washed twice with HKR; the cells were then prepared for culture. Cells prepared by this method routinely exclude trypan blue dye greater than 95% and contain approximately 5% contamination with fasciculata-reticularis cells (as determined by visual inspection of the size difference between the two cell types).

Culture of adrenal glomerulosa cells

Isolated porcine adrenal glomerulosa cells were centri-

fuged and resuspended with DMEM/Ham's F-12 (1:1, volume:volume) containing 10% horse serum, 2% fetal calf serum, 2 nM insulin, 1 mM ascorbic acid, 1.2 μM transferrin, 1 μM tocopherol, 50 nM sodium selenite, 50 μM metyrapone, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin and 25 μg/mL of amikin. Cells were then transferred to a fibronectin-treated 24-well culture cluster at a concentration of 0.5-1 $\times 10^6$ cells/well and maintained in a humidified atmosphere of 95% air/5% CO_2 at 37℃. After 24 hours, cells were washed twice with DMEM/Ham's F-12 medium containing 20 mM HEPES (pH 7.3) and 0.1% BSA, then cultured in serum-free medium for another 18-20 hours before stimulation.

Measurement of aldosterone secretion

Cells maintained in a serum-free medium for 18-20 hours were washed twice with DMEM/Ham's F-12 medium containing 20 mM HEPES (pH 7.3) and 0.1% BSA, then stimulated for 3 hrs with either EGF, AII, or EGF and AII together. After stimulation, the supernatant was collected and the concentration of aldosterone was determined with a radioimmunoassay kit.

Western blot analysis of cellular proteins using antiphosphotyrosine antibodies

Cells maintained in serum-free medium for 18-20 hours were washed twice with DMEM/Ham's F-12 medium containing 20 mM HEPES (pH 7.3) and 0.1% BSA, then incubated with 0.25 mL of DMEM/Ham's F-12 medium containing 20 mM HEPES (pH 7.3) and 0.1 % BSA at 37℃ for 30 min. For time-dependent tyrosine phosphorylation, 10 ng/mL of EGF was added at timed intervals. For the stimulation of tyrosine phosphorylation, cells were treated with various concentrations of EGF and AII for 2 min. Stimulation was terminated by aspirating the medium. Each well was then washed twice with 400 μL of ice-cold phosphate-buffered saline and treated with 100 μL of ice-cold lysis buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 10% (vol/vol) glycerol, 50 mM NaF, 1 mM phenylmethylsulfonylfluoride, 1 mM Na_3Vo_4, and 10 μg/mL leupeptin) for 20 min on ice. Lysed cells were transferred to microcentrifuge tubes and centrifuged at 10,000 *g* for 10 min at 4℃. After determining the concentration of the protein with a BCA protein quantification kit, 20 μg of each proteins was loaded onto 10% SDS-polyacrylamide gel and separated for 1 hr at 100 V. Separated proteins were then transferred to nitrocelluose filters for 1 hr at 80 V. After blocking with 2% (wt/vol) BSA, filters were probed with monoclonal antibodies to phosphotyrosine. The immune complex was detected by a electrochemiluminescent system and autoradiography.

Measuring generation of inositol phosphates

The PLC activity was determined by measuring the formation of inositol phosphates according to the method described by Downes et al. (18) with a slight modification. In brief, cells were labeled with [^{3}H]*myo*-inositol. When maintained in serum-free medium, 12 μCi/mL of [^{3}H]*myo*-inositol was added to each well and labeled for 18-20 hrs. After labeling, [^{3}H]*myo*-inositol was removed by aspirating the labeling medium and washing twice with DMEM/Ham's F-12 medium containing 20 mM HEPES (pH 7.3) and 0.1% BSA. Cells were then incubated in 300 μL of DMEM/Ham's F-12 medium containing 20 mM HEPES (pH 7.3), 0.1% BSA and 15 mM LiCl for 10 min at 37℃. EGF or AII-or both-was/were added to the medium and incubated for a further 10 min. The stimulation was terminated by aspirating the medium and cells were rinsed twice with ice-cold PBS, then treated with 0.6 mL of ice-cold 5% perchloric acid for 20 min. They were scraped into microcentrifuge tubes and the cellular debris was pelleted by centrifugation. The supernatants were saved and 0.55 mL was diluted with 15 mL DW and loaded onto a column containing 2 mL of Dowex AG-1X8 anion exchange resin. The column was washed with 8 mL DW and 10 mL of 60 mM sodium formate/5 mM sodium borate solution, and inositol phosphates were eluted with 5 mL of 1M ammonium formate/0.1 mM formic acid solution. The radioactivity of the elutes was measured with a Beckman β counter.

Statistical analysis

The experimental results were analyzed with a SAS statistical package, and expressed as mean±standard deviation. Comparisons were made using unpaired Student's t test or ANOVA (Sheffe's method).

RESULTS

Stimulation of aldosterone secretion by EGF

To investigate the effect of EGF on aldosterone secretion in cultured porcine adrenal glomerulosa cells, these cells were treated 3 hrs after stimulation with increasing concentrations of EGF and the concentration of aldosterone in culture supernatant was measured 3 hours after stimulation. EGF stimulated aldosterone secretion and statistically significant stimulation was observed at concentrations of 10 ng/mL or more of EGF (Fig. 1).

Effect of EGF on AII-induced aldosterone secretion

To determine whether EGF had any synergistic, addi-

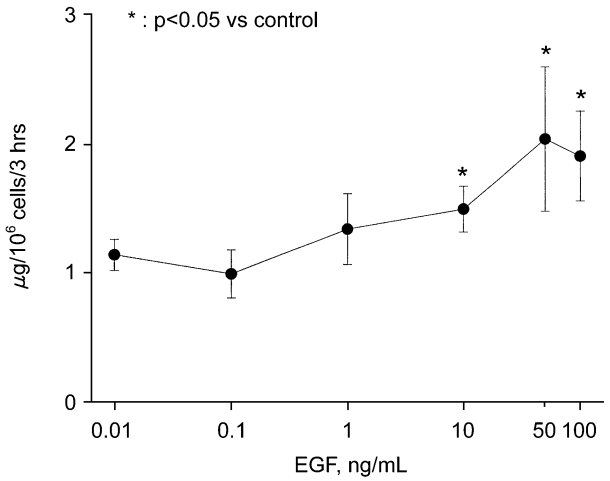

Fig. 1. The effects of increasing concentrations of epidermal growth factor(EGF) on aldosterone secretion in cultured porcine adrenal glomerulosa cells. Cells were incubated in medium containing the indicated concentrations of EGF. Each value represents mean±S.D. of five separate experiments. * P<0.05 vs. basal

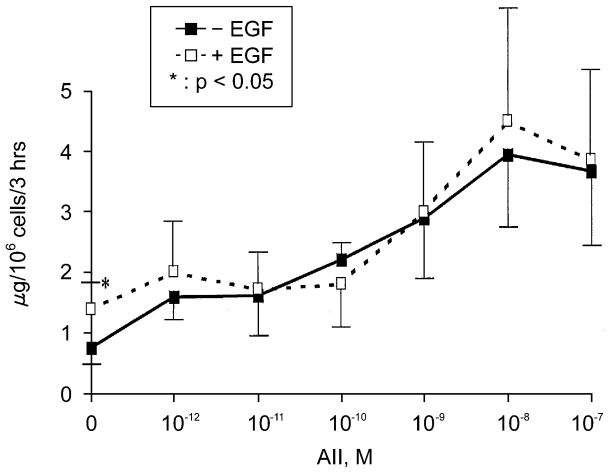

Fig. 2. The effects of EGF on basal and AII-stimulated aldosterone secretion in cultured porcine adrenal glomerulosa cells. Cells were incubated in a medium containing the indicated concentrations of AII in the absence (■) or presence (□) of 10 ng/mL EGF. Each value represents mean±S.D. of six separate experiments. *P<0.05 vs. basal

tive or inhibitory effect on AII-induced aldosterone secretion, adrenal glomerulosa cells were concurrently stimulated with EGF and AII. For all experiments, 10 ng/mL EGF was added to increasing concentrations of AII. EGF alone increased aldosterone secretion (0.76±0.54 vs. 1.38±0.77 ng/10^6 cells/3 hrs), but the addition of EGF neither increased nor decreased the aldosterone secretion induced by different concentrations of AII (Fig. 2).

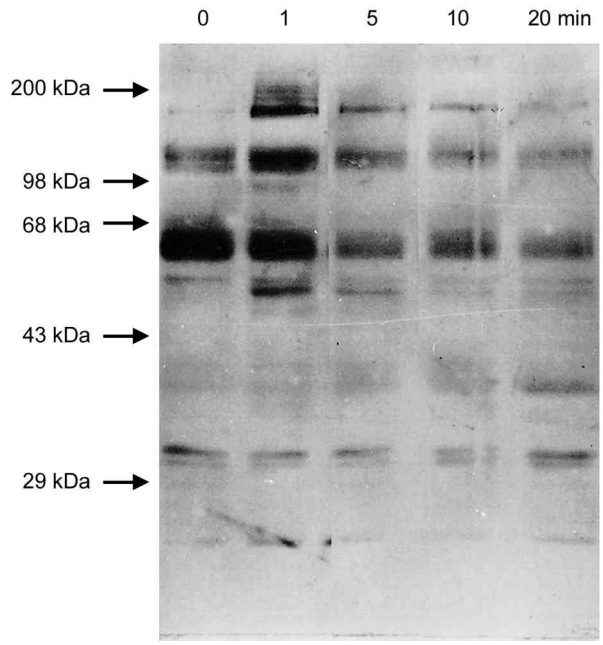

Fig. 3. Time course of EGF or AII-induced protein tyrosine phosphorylation in cultured porcine adrenal glomerulosa cells. Cells were treated with vehicle (lane 1), 10 ng/mL EGF or 10^{-8} M AII for 2 min at 37℃ as described in the method section. Stimulation was then terminated and cells were solubilized. Total cellular proteins were separated on 10 % SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The immune complex was probed with anti-phosphotyrosine antibodies and visualized by the technique of enhanced chemiluminescence. The positions of prestained molecular mass markers are indicated.

Tyrosine phosphorylation of cellular proteins induced by EGF or AII

The treatment of adrenal glomerulosa cells with 10 ng/mL of EGF provoked a rapid and transient tyrosine phosphorylation of several proteins (Fig. 3). EGF treatment not only enhanced the tyrosine phosphorylation of proteins already phosphorylated in the basal state (105 kDa and 60 kDa), but also evoked tyrosine phosphorylation of new proteins (170 kDa and 50 kDa). This EGF-induced tyrosine phosphorylation peaked at 1 min, then decreased rapidly, and returned to near basal level after 20 min. To determine the effect of varying amounts of EGF, cells were treated for 2 min and the extent of tyrosine phosphorylation was analyzed by immunoblot. Tyrosine phosphorylation continued to increase until EGF concentration reached 50 ng/mL, then decreased when 100 ng/mL of EGF were added (Fig. 4). AII (1×10^{-8}M) enhanced tyrosine phosphorylation of several proteins, which had already been phosphorylated in the basal state, but failed to induce the phosphorylation of new proteins.

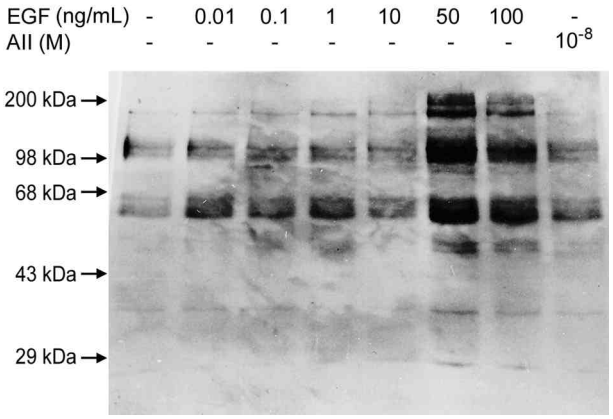

Fig. 4. The effects of increasing concentrations of EGF on protein tyrosine phosphorylation in cultured porcine adrenal glomerulosa cells. Cells were treated with 0-100 ng/mL EGF for 2 min at 37℃ and tyrosine phosphorylation was detected with anti-phosphotyrosine antibodies. The positions of prestained molecular mass markers are indicated.

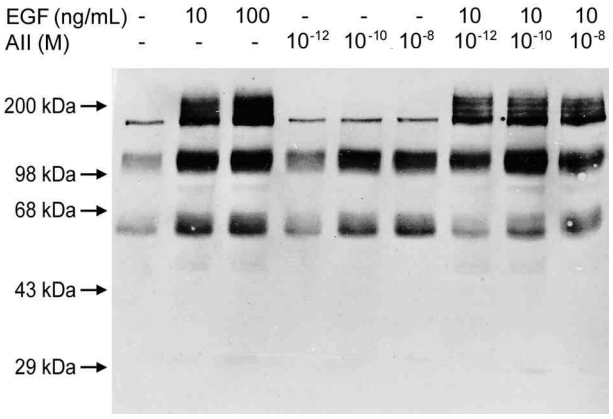

Fig. 5. The effects of AII alone or both EGF and AII on protein tyrosine phosphorylation in cultured porcine adrenal glomerulosa cells. Cells were treated with the indicated concentrations of AII with or without 10 ng/mL EGF for 2 min at 37℃ as described in the method section.

In order to determine whether EGF-induced tyrosine phosphorylation was affected by AII, cells were treated with 10 ng/mL of EGF and different concentrations of AII. Although AII alone elevated tyrosine phosphorylation of cellular proteins in a dose dependent manner (10^{-12} M to 10^{-8} M), it neither increased nor decreased tyrosine phosphorylation elicited by EGF (Fig. 5).

Formation of inositol phosphates after stimulation with EGF or AII

Because AII-stimulated aldosterone secretion is known to be mediated by the PLC action, we investigated

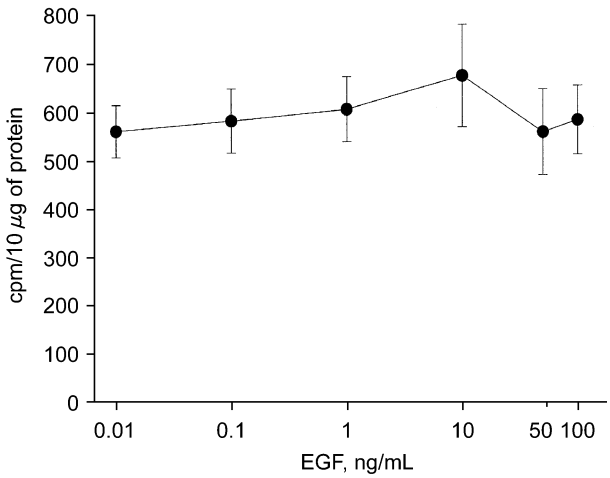

Fig. 6. Lack of EGF effects on total inositol phosphates formation in cultured porcine adrenal glomerulosa cells. Cells were labeled with 12 mCi/mL [^{3}H]*myo*-inositol for 20 hrs. After labeling, they were treated with the indicated concentrations of EGF for 10 min at 37℃ in the presence of 15 mM LiCl. On termination of the reaction, samples were assayed for the total accumulation of [^{3}H] inositol phosphates by anion-exchange chromatography. Each value represents the mean±S.D. of four separate experiments.

whether EGF-induced aldosterone secretion was associated with the activation of PLC. To measure PLC activity, cells were labeled with [^{3}H]*myo*-inositol and the formation of inositol phosphates after stimulation with EGF for 10 min was measured. Although EGF is known to induce tyrosine phosphorylation of PLC-γ1, activating it in various cell systems, we did not observe any increase of inositol phosphates at various concentrations of EGF at which EGF had elicited tyrosine phosphorylation of many proteins (Fig. 6). At a concentration of 10^{-9} M or more, however, AII stimulated the formation of inositol phosphates (Fig. 7), but the addition of 10 ng/mL of EGF at various concentrations did not affect AII-induced activation of PLC at any concentrations.

DISCUSSION

In the present study, we demonstrated that EGF stimulated aldosterone secretion in cultured porcine adrenal glomerulosa cells through tyrosine phosphorylation, but probably not through the activation of PLC. We also showed that although EGF alone stimulated aldosterone secretion, the addition of EGF neither increased nor decreased AII-induced aldosterone secretion, which suggested that interaction between the AII- and EGF-stimulated signaling pathways obliterated EGF-induced aldosterone secretion. The same was true for EGF-provoked

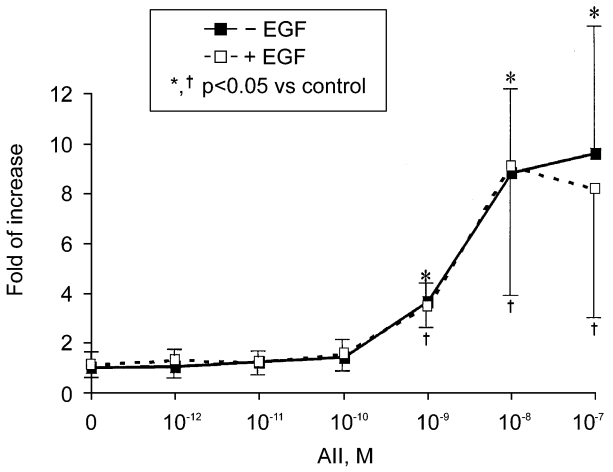

Fig. 7. Effects of AII alone or both EGF and AII on total inositol phosphates formation in cultured porcine adrenal glomerulosa cells. Cells were labeled with 12 mCi/mL [^{3}H]*myo*-inositol for 20 hrs and stimulated with the indicated concentrations of AII in the absence (■) or presence (□) of 10 ng/mL EGF for 10 min. Data are expressed as the folds of increase from the basal state, defined as 1 fold. Each value represents the mean±S.D. of 2-4 separate experiments performed in duplicate. *,† $P<0.05$ vs. basal

tyrosine phosphorylation, which remained the same whether or not AII was added.

Although EGF has been reported to affect steroid hormone biogenesis in gonadal cells (8-10) and stimulate cortisol secretion in adrenal cortical cells (12, 19), its role in aldosterone secretion is not fully understood. Natarajan et al. (7) reported that stimulation of rat or human glomerulosa cells with EGF led to the secretion of aldosterone, and this stimulatory effect was further increased by the simultaneous addition of AII. Our results showed that the effect of EGF on aldosterone secretion in porcine adrenal glomerulosa cells was similar.

Although EGF exerts its effect through a variety of pathways (20-26), tyrosine phosphorylation is the most important signaling pathway (27-30). Once EGF binds to its receptor, activated EGF receptors dimerize and autophosphorylate their tyrosine residues. By recognizing these residues, many cellular proteins containing SH2 domain, such as PLC-γ, Grb2, phosphatidylinositol-3-kinase, or GTPase activating protein, then move to the EGF receptor; this phosphorylates these SH2-containing proteins and thus activates or inactivates them (31). Antiphosphotyrosine immunoblotting revealed the 170 kDa-band. Though immunoblot assay using anti-EGF receptor Ab was not performed, we speculated that this 170 kDa-band might be an EGF receptor; its molecular weight corresponded to the known molecular weight of the EGF receptor, its phosphorylation peaked at 1 min after stimulation, and it was not observed when cells were stimulated with AII. Furthermore, tyrosine phosphorylation increased as the amount of EGF increased, and peaked at 50 ng/mL of EGF. EGF-induced aldosterone secretion showed a similar increase, suggesting that EGF-induced tyrosine phosphorylation might be responsible for EGF-stimulated aldosterone secretion. We did not scrutinize the effect of tyrosine kinase inhibitors on EGF-induced aldosterone secretion, therefore we concluded that EGF-induced tyrosine phosphorylation was most probably the mechanism involved in the stimulation of aldosterone secretion by EGF.

If this is so, our next question was whether or not PLC activation was involved in this process. In most cellular systems, EGF is known to be an important signal to activate PLC-γ (32-36), but in cells such as Swiss 3T3 fibroblasts (37), BALB/c/3T3 fibroblasts (38), Chinese hamster fibroblasts (39) and T-cell hybridoma (40), it did not stimulate tyrosine phosphorylation of PLC-γ. This was the case in our experiment. EGF definitely induced tyrosine phosphorylation of many intracellular proteins in porcine adrenal glomerulosa cells, but we observed no evidence of PLC activation, as measured by the formaiton of inositol phosphates. The discrepancy between EGF-induced tyrosine phosphorylation and the lack of inositol phosphates formation can be explained in several ways. First, if porcine adrenal glomerulosa cells contain PLC-β isoforms but not PLC-γ isoforms, EGF can induce tyrosine phosphorylation but cannot stimulate the formation of inositol phosphates. We did not perform experiments to look at the presence of PLC-γ isoforms in this cell because antibodies to PLC-γ2 were not commercially available. However, although we did not observe PLC-γ in this cell, it cannot be said that it was definitely not present. Based on the experiments using anti-PLC-β1, anti-PLC-γ1 and anti-PLC-δ1 antibodies, Rhee et al. (41) showed that while PLC-β1 and PLC-δ1 may be lost when cells are transformed to permanent cell lines, every cell line studied in their experiment retained PLC-γ1. Because this is activated mainly by growth factors, it is speculated that it may be essential for cell growth. This implies that PLC-γ is a kind of house-keeping enzyme, required by every cell for growth and proliferation. It cannot, therefore, be reliably concluded that the absence of inositol phosphates formation was due to the absence of PLC-γ. Second, it is possible that too little inositol phosphates was formed by this to have been detected by conventional assay. If, however, PLC really mediated the effect of EGF on aldosterone secretion, the extent of PLC activation would be associated with the aldosterone secretion. In AII-mediated aldosterone secretion, the generation of inositol phosphates increased about 18 times (at 10^{-8} M of AII) but aldosterone secretion increased by a factor of only six. In

our study, EGF treatment caused aldosterone secretion to approximately double, but we detected no increase in the formation of inositol phosphates. According to AII-induced activation, we should have observed an approximately six-fold increase in inositol phosphates formation, and this should have been easily detected. Third, EGF-induced tyrosine phosphorylation might not have activated PLC-γ, and aldosterone secretion was mediated by another molecule phosphorylated by EGF receptor. Natarajan et al. (7) suggested that EGF-induced aldosterone secretion might be mediated by increased DAG. Since this increase can be achieved not only by the activation of PLC or phospholipase D, or by de novo synthesis of DAG, their data implied that aldosterone secretion can be induced without the activation of PLC. This PLC-independent increase of DAG might be a plausible mechanism of aldosterone secretion in this particular cell. Availability of antibodies to PLC-γ1 or PLC-γ2 would be critical to prove this possibility.

AII receptor belongs to the seven transmembrane receptor family, which couples to G protein and thus activates PLC or adenylate cyclase. Several investigators have recently demonstrated that AII stimulated tyrosine phosphorylation of various proteins; PLC-γ1 through the recruitment of soluble tyrosine kinases in vascular smooth muscle cells (13) and mesangial cells (14). Furthermore, recent reports have insisted that AII stimulated aldosterone secretion through the activation of tyrosine kinases in adrenal glomerulosa cells (15, 16) and our data also suggested that AII enhanced tyrosine phosphorylation of several proteins. For two reasons, it could not be concluded that tyrosine phosphorylation played an important role in aldosterone secretion. First, AII did not induce tyrosine phosphorylation of new proteins; rather it enhanced tyrosine phosphorylation of proteins already phosphorylated in the basal state. Second, although tyrosine phosphorylation of several proteins was induced by AII, these same proteins were also phosphorylated by EGF. Furthermore, EGF induced a much higher degree of phosphorylation than AII did. These results suggest that AII can induce tyrosine phosphorylation of several proteins. Phosphorylation, however, did not seem to be the principal mechanism involved in aldosterone secretion; although it can induce stronger phosphorylation of same proteins, EGF failed to activate PLC. It therefore seems reasonable to conclude that a G protein-mediated signal, rather than tyrosine phosphorylation, is the main pathway involved in AII-mediated aldosterone secretion.

In our study, EGF stimulated aldosterone secretion, though costimulation of cells with EGF and AII had no additive or synergistic effect. Because the responsiveness of glomerulosa cells to external stimuli varies considerably from preparation to preparation, the precise determination of EGF-induced aldosterone secretion induced by EGF might be somewhat difficult. Furthermore, since the level of this was only 30% of the level of AII-induced aldosterone secretion (data not shown), a small increase in EGF-induced aldosterone secretion might be masked by a larger increase in aldosterone secretion stimulated by a stronger AII-mediated signal. However, our observations showed repeatedly that the addition of EGF did not affect AII-stimulated aldosterone secretion, no matter the concentration of AII. If this is the case, our results suggest a close interaction between AII- and EGF-mediated signaling pathways. If these were independent, aldosterone secretion would be additive, unless substrates for synthesis were limited. This was not the case, however. No additive effect was observed, even at low concentrations of AII at which substrate availability should be sufficient. We therefore suggest that once the AII-mediated pathway is activated, it inhibits EGF-mediated aldosterone secretion, as was observed in the phosphorylation of cellular proteins by AII or EGF. Although AII alone enhanced tyrosine phosphorylation of 150 kDa- and 60 kDa-proteins, the addition of AII did not alter the phosphorylation pattern induced by EGF alone. This in turn suggests that once EGF is activated, AII might no longer have any effect.

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