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Angiotensin II Activates Cholesterol Ester Hydrolase in Bovine Adrenal Glomerulosa Cells through Phosphorylation Mediated by p42/p44 Mitogen-Activated Protein Kinase

NADIA CHERRADI, BRUNO PARDO, ANDREW S. GREENBERG, FREDRIC B. KRAEMER, AND ALESSANDRO M. CAPPONI

Division of Endocrinology, Diabetology, and Nutrition (N.C., B.P., A.M.C.), Department of Internal Medicine, Faculty of Medicine, CH-1211 Geneva, Switzerland; Jean Meyer United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University (A.S.G.), Boston, Massachusetts 02111; and Veterans Affairs Palo Alto Health Care System and Stanford University (F.B.K.), Stanford, California 94305

In adrenal glomerulosa cells, the stimulation of aldosterone biosynthesis by angiotensin II (Ang II) occurs via activation of the Ca^{2+} messenger system, increased expression of the steroidogenic acute regulatory protein, and enhanced transfer of cholesterol to the inner mitochondrial membrane. We examined here whether Ang II affects the activity of cholesterol ester hydrolase (CEH), also named hormone-sensitive lipase, the enzyme recruiting cholesterol from intracellular pools, in bovine adrenal glomerulosa cells. In bovine adrenal tissue, CEH activity was detected with characteristics similar to those reported in other tissues (Michaelis constant = 46.3 ± 6.7 μM, n = 3; maximal velocity = 1 nmol/mg·min). This activity was significantly enhanced in isolated bovine glomerulosa cells challenged for 2 h with 10 nM Ang II (to 149 ± 11% of controls, n = 3). Similarly, 25 μM forskolin raised CEH activity to 151 ± 5% of controls (n = 3). This increase in activity of CEH was not due to an increase in the amount of enzyme protein but was associated with an increased phosphorylation of the enzyme to 337 ± 33% of controls (n = 9, $P < 0.0001$). Potassium ion (K^+) and forskolin also stimulated [^{32}P]orthophosphate incorporation, although to a lesser extent (to 157 ± 18% and 186 ± 25% of controls, respectively). On SDS-PAGE, the majority of this radioactivity was associated with a species of 172 kDa, corresponding to a CEH dimer. Both Ang II-induced CEH phosphorylation and pregnenolone production were significantly reduced (to 47 ± 6% and 50 ± 8% of controls with Ang II alone, respectively) in the presence of PD098059, an inhibitor of p42/p44 MAPK. Indeed, Ang II challenge led to a rapid ^{32}P incorporation into p42/p44 MAPK. These results demonstrate that, in addition to its known effects on intramitochondrial cholesterol transfer, Ang II also promotes aldosterone biosynthesis by rapidly increasing cholesterol supply to the outer mitochondrial membrane. (*Endocrinology* 144: 4905–4915, 2003)

IN MAMMALS, ALL STEROID hormones are synthesized from a common precursor, cholesterol. It is well known that hormonal stimulation of steroid-producing cells results in prompt mobilization of cholesterol esters from intracellular lipid droplets (1, 2). These cholesterol esters are hydrolyzed to free cholesterol by the cholesterol ester hydrolase enzyme (CEH), also named hormone-sensitive lipase, HSL (see below). Cholesterol is then conveyed to mitochondria, in which it is transferred to the side chain cleavage enzyme system in the inner mitochondrial membrane. The latter step, which is rate limiting in steroidogenesis, depends on the action of the steroidogenic acute regulatory protein (StAR) (3).

The regulation of CEH activity through cAMP-dependent mechanisms has been extensively studied (4–9). In the adrenal cortex, ACTH induces marked reductions of cholesterol ester levels with a concomitant increase in CEH activity (2). Like HSL (10–12), its homolog in adipose tissue, CEH is activated *in vitro* after phosphorylation by cAMP-dependent protein kinase (PKA) (13–16). In adrenal glomerulosa cells, the calcium (Ca^{2+}) messenger system mediates the action of the two main physiological activators of aldosterone biosynthesis, angiotensin II (Ang II), and extracellular K^+. Ang II receptor activation leads, on the one hand, to protein kinase C (PKC) activation (17) and, on the other hand, to the release of Ca^{2+} from intracellular stores (18) and to Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels (19–21). K^+ stimulates aldosterone synthesis by enhancing Ca^{2+} influx through voltage-operated channels (22).

We have previously shown that Ang II increases both the expression of the StAR protein and the transfer of cholesterol from the mitochondrial outer membrane to the inner membrane (23, 24). This transfer, however, does not lead to a depletion of the cholesterol pool in the outer membrane, suggesting that Ang II also promotes cholesterol supply to the mitochondria from intracellular lipid droplets, whereas calcium *per se* promotes only intramitochondrial cholesterol transfer (23). We therefore examined in the present study whether the Ca^{2+}-mobilizing agonists Ang II and K^+ affect the activity of cholesterol ester hydrolase in bovine adrenal glomerulosa cells. Our data show that Ang II and K^+ are indeed effective in stimulating CEH activity. We also provide evidence that Ang II and K^+ activate CEH through distinct signaling pathways.

Abbreviations: Ang II, Angiotensin II; Ca^{2+}, calcium; CaMK, calmodulin-dependent protein kinase; CEH, cholesterol ester hydrolase; HSL, hormone-sensitive lipase; PKA, protein kinase A; PKC, protein kinase C; SDS, sodium dodecyl sulfate; StAR, steroidogenic acute regulatory protein.

Materials and Methods

Materials

Cholesteryl [1-^{14}C]oleate, [9,10-^{3}H]oleic acid, and [7-^{3}H]pregnenolone were obtained from DuPont NEN Life Science Products (Boston, MA). [Ile^{5}]Ang II was purchased from Bachem (Bubendorf, Switzerland), and [^{32}P]orthophosphate from Hartmann Analytic (Braunschweig, Germany). Ionomycin and KN93 were obtained from Calbiochem (Lucerne, Switzerland). Calphostin C, PD098059, and SB203580 were purchased from Alexis Biochemicals (Läufelfingen, Switzerland). Antipregnenolone antiserum was obtained from Biogenesis Ltd. (Poole, UK). Fatty acid-free BSA, cholesteryl oleate, oleic acid, dithioerythritol, and all other chemicals used were purchased from Sigma (St. Louis, MO) or Fluka (Buchs, Switzerland). Polyclonal antibodies against rat hormone-sensitive lipase, the homolog of CEH in adipocytes, were generated in rabbits as previously reported (25). Protein A/G agarose and anti-p42 and -p44 MAPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against the phosphorylated forms of p42/p44 MAPK were from New England BioLabs (Beverly, MA).

Bovine adrenal zona glomerulosa tissue and cell preparation

Bovine adrenal glands were obtained from a local slaughterhouse and sliced in half lengthwise on ice. Zona fasciculata and medulla were carefully removed. Zona glomerulosa was scraped from the capsule and homogenized in ice-cold 5 mM Tris-HCl buffer (pH 7.4) containing 275 mM sucrose. The homogenate was centrifuged at 200 × *g* for 15 min at 4 C to pellet large debris and nuclei. The postnuclear supernatant was used to determine optimal enzymatic conditions and kinetic constants for CEH activity.

Primary cultures of glomerulosa cells were prepared by enzymatic dispersion with dispase and purified on Percoll density gradients as described elsewhere (26). Cells were kept in serum-free medium for 1 d before experiments, which were performed on the third day of culture. At the end of the stimulation period, cells were homogenized as reported previously (27). Protein content was determined by the method of Bradford using AG reagent (Bio-Rad Laboratories, Glattbrugg, Switzerland) and BSA as a standard.

Cholesterol ester hydrolase assay

CEH activity was measured in the postnuclear supernatant of tissue or cultured cells homogenates essentially as described elsewhere (7). Cholesteryl [1-^{14}C]oleate and unlabeled cholesteryl oleate were dissolved in benzene, dried under N_2, and resuspended in ethanol with vortexing and heating at 37 C. Then 50 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM dithioerythritol and 25 mg/ml BSA were slowly added to the ethanol suspension to make the substrate solution. The conversion of cholesteryl [1-^{14}C]oleate (30 μM, 8000 cpm/nmol) to [1-^{14}C]oleic acid was started by adding aliquots of the postnuclear supernatant (50–100 μg protein) to 0.4 ml substrate solution and was carried out for 90 min at 37 C. The reaction was stopped by adding 50 μl of 1 N NaOH followed by 1.5 ml chloroform:methanol:benzene (2:2.4:1) and unlabeled oleic acid (45 μg) to increase extraction efficiency. Tubes were vortexed for 15 sec and then centrifuged at 1500 × *g* for 10 min. A 0.5-ml aliquot of the top aqueous phase was removed for scintillation counting. [9,10-^{3}H]oleic acid was used to determine extraction efficiency, which ranged from 70% to 80%.

Radiolabeling of glomerulosa cells and CEH immunoprecipitation

Glomerulosa cells in primary culture (15×10^6 cells/10 cm petri dish) were prelabeled for 60 min in phosphate-free Krebs-Ringer bicarbonate buffer containing 3% BSA and 200 μCi/ml [^{32}P]orthophosphate before exposure to 10 nM Ang II, 12 mM K^+, 2 μM ionomycin, or 25 μM forskolin for 2 h at 37 C. In experiments designed to identify the protein kinases involved in CEH phosphorylation, specific inhibitors were added in the incubation medium 30 min before the agonists. At the end of the stimulation period, cells were washed twice with ice-cold PBS and lysed in 0.5 ml ice-cold lysis buffer (150 mM NaCl, 3% Triton X-100, 0.1% sodium *N*-lauroyl sarcosinate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 mM NaF, 100 nM okadaic acid, and 200 μM sodium orthovanadate). Samples were briefly sonicated and centrifuged at 10,000 × *g* for 15 min. The floating lipid layer was carefully removed. An aliquot of 400 μl was precleared with 20 μl protein A/G agarose mixture and incubated with 1 μg/ml rabbit polyclonal anti-HSL antibody at 4 C for 12 h. The immune complex was isolated by adding 30 μl protein A/G mixture for 2 h at 4 C and then centrifuging at 4000 rpm for 5 min. The pellet was washed four times with a buffer containing 20 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 mM NaF, 100 nM okadaic acid, and 200 μM sodium orthovanadate. The pellet was resuspended in 20 μl electrophoresis sample buffer containing 8 M urea, boiled for 5 min, and analyzed on 10% polyacrylamide gels. The gels were stained, dried, and exposed to Hyperfilm MP (Amersham, Zurich, Switzerland) at −80 C for 24 to 36 h.

SDS-PAGE analysis and immunoblotting

SDS-polyacrylamide (0.1%; 10%) gel electrophoresis was performed according to Laemmli (28). Aliquots from postnuclear supernatants were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (29), which was incubated in blocking buffer (PBS, 0.2% Tween 20, 5% nonfat dry milk) for 2 h, and exposed to 0.5 μg/ml rabbit polyclonal anti-HSL antibody at 4 C for 12 h. The membrane was thoroughly washed with PBS/Tween buffer (3 × 10 min) and then incubated for 1 h with horseradish peroxidase-labeled goat antirabbit IgG. The nitrocellulose sheet was then washed 4 × 15 min and the antigen-antibody complex revealed by enhanced chemiluminescence using the Western blotting detection kit and Hyper-ECL film (Amersham). p42/p44 MAPK were immunodetected in glomerulosa cells lysates after SDS-PAGE analysis and transfer on polyvinylidene fluoride membranes (Millipore, Volketswil, Switzerland). The membranes were probed with antiphosphorylated MAPK or antitotal MAPK antibodies according to the instructions of the manufacturer.

Steroid measurement

Pregnenolone was measured in the medium of glomerulosa cells cultured in 24-well plates (500,000 cells/well) by RIA using a commercially available antiserum (Biogenesis Ltd.). WIN 19758 (5 μM) was included in the incubation medium to prevent conversion of pregnenolone into progesterone. Pregnenolone production was expressed per milligram cellular protein.

Analysis of data

Results are expressed as means ± SEM. The mean values were compared by ANOVA using Fisher's test. A value of $P < 0.05$ was considered as statistically significant. Quantitation of autoradiograms was performed using a Molecular Dynamics (Sunnyvale, CA) computing densitometer and ImageQuant software.

Results

Characterization of bovine adrenal zona glomerulosa CEH activity

To ensure accurate measurements of enzyme activity in bovine adrenal zona glomerulosa cells, optimal conditions for CEH assay were first determined through time-course and concentration-response experiments for both protein and substrate concentrations. Cholesterol ester hydrolysis was linear for incubations up to 180 min using 100 μg protein and 30 μM cholesteryl oleate (Fig. 1A). The concentration dependency for protein showed that the reaction was linear up to 150 μg protein using 30 μM cholesteryl oleate and a 90-min incubation time (Fig. 1B). To measure saturating substrate concentrations and determine Michaelis constant and maximum velocity, 50–100 μg protein were incubated with

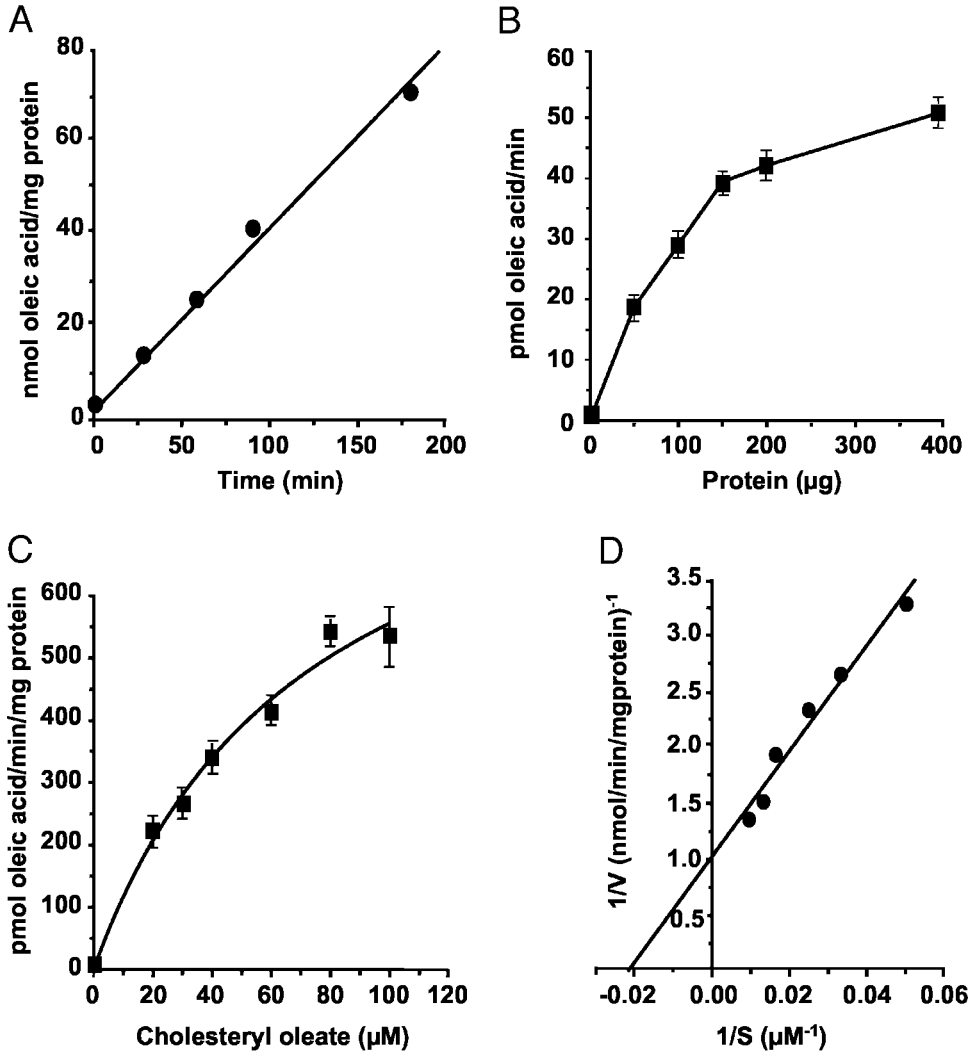

FIG. 1. Characterization of bovine adrenal zona glomerulosa CEH activity. CEH activity was measured in the postnuclear supernatant of zona glomerulosa homogenates as described in *Materials and Methods*. A, Time course of cholesteryl oleate hydrolysis. Assays were conducted with 100 μg protein and 30 μM cholesteryl oleate. B, Effect of protein concentration on cholesteryl oleate hydrolysis (30 μM cholesteryl oleate, 90-min incubation time at 37 C). C, Effect of substrate concentration. The conversion of cholesteryl [1-^{14}C]oleate (20–100 μM) to [1-^{14}C]-oleic acid was started by adding 50–100 μg protein and carried out for 90 min at 37 C. D, Lineweaver-Burk transformation of original data. Each data point is the mean ± SEM of four separate tissue preparations, with duplicate determinations performed for each time, amount of protein, and substrate concentration.

increasing concentrations of cholesteryl oleate. The reaction velocity increased linearly up to 40 μM substrate (Fig. 1C). The Michaelis's constant and maximum velocity values of CEH for cholesteryl oleate were 46.3 μM and 1 nmol/min·mg protein, as assessed after Lineweaver-Burk transformation of original data (Fig. 1D). The assays conducted with glomerulosa cells in primary culture were subsequently performed with 30 μM substrate for 90 min at 37 C.

Ang II, K^+, and forskolin significantly increase CEH activity

We previously reported that stimulation of glomerulosa cells with Ang II activates the rate-limiting step of aldosterone biosynthesis, namely cholesterol transfer from the mitochondrial outer membrane to the inner membrane (23). However, in contrast to what is observed under an ionomycin-generated cytosolic calcium clamp, Ang II does not deplete the cholesterol pool in the outer membrane (23), suggesting that the hormone also promotes cholesterol supply to mitochondria from intracellular lipid droplets, possibly through the activation of cholesterol ester hydrolysis. To test this hypothesis, we examined the effect of Ang II and K^+ on CEH activity. Glomerulosa cells in primary culture were stimulated with 10 nM Ang II or 12 mM K^+ for 2 h. The postnuclear supernatant of cell homogenates was used to measure CEH activity. In a typical experiment, oleic acid production was 45 ± 5, 68 ± 1, 52 ± 2, and 72 ± 3 pmol/min·mg protein for controls, Ang II-, K^+-, and forskolin-stimulated cells, respectively (n = 3). As shown in Fig. 2, Ang II and K^+ increased significantly CEH activity to 149 ± 11 (n = 3, $P < 0.05$) and 129 ± 3% (n = 3, $P < 0.01$) of control values, respectively. When CEH activity was determined in immunoprecipitates, in the absence of protease and phosphatase inhibitors, Ang II increased CEH activity similarly, to 154% of controls (data not shown). In contrast, an ionomycin-mediated increase in cytosolic calcium concentration to 600–800 nM had no significant effect on CEH activity (112 ± 17% of controls, n = 3, data not shown). As shown previously in mouse Y1 adrenal tumor cells (30), raising intracellular cAMP levels with forskolin increased CEH activity in glomerulosa cells to 151 ± 4.8% of controls (n = 3, $P < 0.01$, Fig. 2). These results suggested that Ang II and K^+ stimulate the supply of cholesterol, the precursor of steroids, from intracellular stores to mitochondria via the activation of CEH.

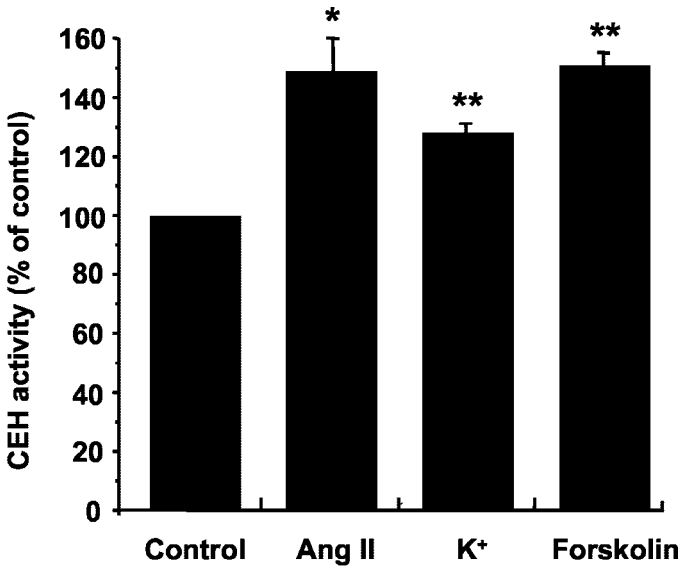

FIG. 2. Ang II, K^+, and forskolin increase CEH-specific activity. Glomerulosa cells in primary culture were stimulated with 10 nM Ang II, 12 mM K^+, or 25 μM forskolin at 37 C for 2 h. Cells were homogenized and the postnuclear supernatant (50–100 μg protein) was used to measure CEH activity. The enzymatic activity of CEH in stimulated cells is expressed as a percentage of the activity measured in control cells. *Bars* represent the mean ± SEM of duplicate determinations from three independent experiments. In a typical experiment, oleic acid production in control cells was 45 ± 5 pmol/min·mg protein, and aldosterone production increased from 91 pmol/mg protein (control) to 1200 pmol/mg protein (Ang II) and 660 pmol/mg protein (K^+). (*, **, Significantly different from controls with $P < 0.05$ and $P < 0.01$, respectively).

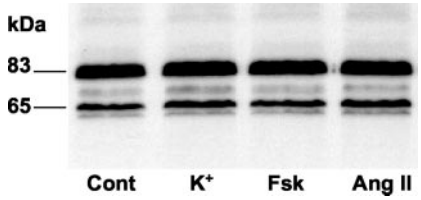

FIG. 3. Ang II and K^+ do not affect CEH protein expression. SDS-PAGE and immunodetection of CEH were performed as described in *Materials and Methods*, using 10 μg protein/lane of the postnuclear supernatant of glomerulosa cells stimulated with 10 nM Ang II, 12 mM K^+, or 25 μM forskolin (Fsk) at 37 C for 2 h.

Ang II, K^+, and forskolin do not affect CEH protein expression

To determine whether the Ang II- or K^+-induced increase in CEH activity was due to an increase in CEH protein expression, equal amounts of postnuclear supernatants from glomerulosa cells were analyzed by Western blot. In control cells, CEH was detected as a protein doublet exhibiting a major immunoreactive protein of approximately 83 kDa and a minor one of 65 kDa (Fig. 3). Neither Ang II nor K^+ and forskolin affected CEH protein content in glomerulosa cells after 2 h of stimulation. Therefore, the increase in CEH activity induced by Ang II and K^+ was not associated with an increase in CEH protein expression.

Ang II, K^+, and forskolin increase CEH phosphorylation level

CEH activity has been shown to be regulated by phosphorylation-dephosphorylation mechanisms of the enzyme after an acute activation of the cAMP messenger system in steroidogenic cells and adipocytes as well as in macrophages (25, 30, 31). To examine whether the calcium-mobilizing agonists Ang II and K^+ affect CEH phosphorylation, CEH was immunoprecipitated from ^{32}P-labeled glomerulosa cells extracts and analyzed by SDS-PAGE. As shown on the Coomassie-blue staining of the gel (Fig. 4A), the major form of CEH immunoprecipitated from cell extracts was a doublet of 172 kDa, possibly corresponding to a dimer of the enzyme or large lipid-protein aggregates. Autoradiography of the gel indicated that a basal phosphorylation level of CEH was detected in control cells and that Ang II, K^+, or forskolin treatment increased ^{32}P incorporation into CEH (Fig. 4B). Densitometric scanning analysis of several independent experiments revealed that Ang II, K^+, and forskolin increased CEH phosphorylation to 337 ± 33 (n = 9, $P < 0.0001$), 157 ± 18 (n = 5, $P < 0.05$), and 186 ± 25% (n = 3, $P < 0.05$) of control values, respectively (Fig. 4C). Upon prolonged exposure of the autoradiogram, the 83- and 65-kDa forms of the enzyme

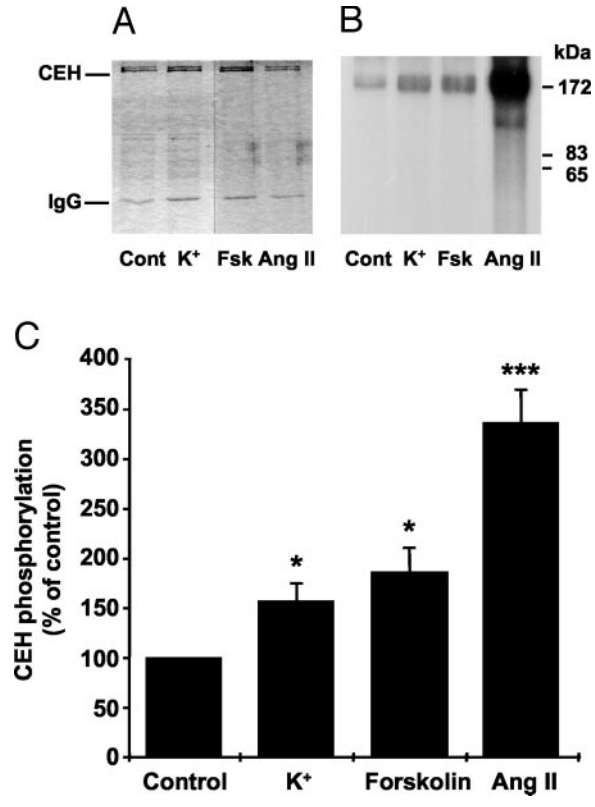

FIG. 4. Ang II, K^+, and forskolin increase CEH phosphorylation. CEH was immunoprecipitated from glomerulosa cells lysates as described in *Materials and Methods* and analyzed by SDS-PAGE and autoradiography. A, Coomassie-blue staining of immunoprecipitated CEH. B, Autoradiography of the gel. C, Densitometric scanning analysis of several independent experiments. The phosphorylation level of CEH in stimulated cells is expressed as a percentage of that measured in control cells (Cont). *, ***, Significantly different from controls with $P < 0.05$ and $P < 0.0001$, respectively; n = 9, 5, and 3 separate experiments for Ang II, K^+, and forskolin (Fsk), respectively.

identified by immunoblotting were also detected and showed the same pattern of phosphorylation as the 172-kDa form (data not shown).

The identity of the 172-kDa doublet was examined in two series of experiments. First, we performed immunoprecipitation of CEH from glomerulosa cell extracts, followed by SDS-PAGE and transfer onto a nitrocellulose membrane. As shown in Fig. 5A, anti-HSL antibodies labeled again the 172-kDa doublet, thus indicating that these species represent CEH as well as the 83- and 65-kDa species. Second, immunoprecipitation of CEH from Ang II-stimulated cell extracts was performed in the presence of increasing amounts of purified HSL fusion protein (Fig. 5B). Following SDS-PAGE, autoradiography of the gel revealed a concentration-dependent decrease of the phosphorylated 172-kDa doublet in the immune complexes, thus indicating that the same antigenic sites are present in HSL and the 172-kDa doublet and clearly identifying these species as CEH.

Figure 6 illustrates the kinetics of Ang II-induced phosphorylation of CEH, indicating that phosphorylation of the enzyme increased during up to 60 min of stimulation and was then maintained for up to 120 min. Not shown here, an ionomycin-mediated cytosolic calcium clamp (600–800 nM) had no effect on CEH phosphorylation in glomerulosa cells, indicating that a sustained increase in cytosolic calcium concentration was not sufficient for activating CEH.

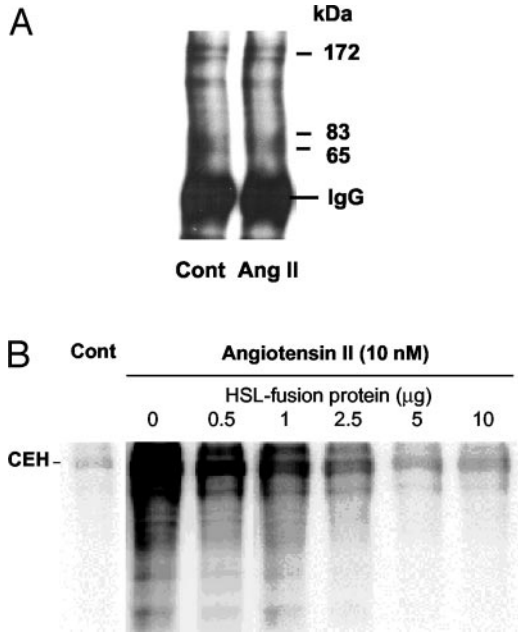

FIG. 5. The 172-kDa doublet is recognized by anti-HSL antibodies. A, Western blot analysis of CEH immune complexes. Immunoprecipitation of CEH from lysates of control or Ang II-stimulated cells was followed by SDS-PAGE and transfer onto a nitrocellulose membrane. The membrane was then probed with anti-HSL antibodies. The weak signal corresponding to the 172-kDa doublet likely is due to a low transfer efficiency for high-molecular-mass proteins (~80% of the 172-kDa doublet remains on the gel, data not shown). B, SDS-PAGE analysis and autoradiography of CEH immune complexes from lysates of control or Ang II-stimulated cells. Then 0.5–10 μg purified HSL fusion protein (52) were added to cell lysates, and immunoprecipitation of CEH was then performed as described in *Materials and Methods*.

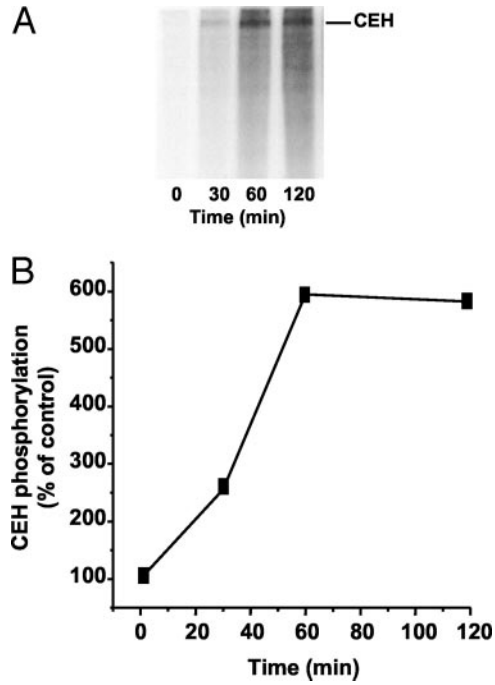

FIG. 6. Time course of CEH phosphorylation by Ang II. A, Glomerulosa cells were stimulated for the indicated times with 10 nM Ang II. Shown is an SDS-PAGE analysis and autoradiography of immunoprecipitated CEH representative of results obtained in two separate experiments. In control cells, basal CEH phosphorylation was detected on prolonged exposure of the autoradiogram. B, Densitometric scanning analysis of the autoradiogram shown in A. The phosphorylation level of CEH in stimulated cells is expressed as a percentage of that measured in control cells.

Altogether these results demonstrated that the Ang II-, K^+-, and forskolin-induced increase in CEH activity is accompanied by an increase in the phosphorylation state of the enzyme.

Neither CaMK nor PKC is involved in Ang II-induced phosphorylation of CEH

To explore potential mechanisms whereby Ang II and K^+ action leads to increased CEH phosphorylation, glomerulosa cells were preincubated with inhibitors of various protein kinases. Several studies support a role of calmodulin and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) as transducers of Ang II and K^+ signals in adrenal glomerulosa cells (32–36). We therefore evaluated the role of CaMK in Ang II- and K^+-induced CEH phosphorylation, using a specific inhibitor, KN93. We first examined the effect of KN93 on the early steps of aldosterone synthesis in bovine glomerulosa cells, namely pregnenolone production (Fig. 7). When glomerulosa cells were challenged with Ang II or K^+ in the presence of increasing concentrations of KN93, a concentration-dependent inhibition of pregnenolone production was observed. However, although KN93 only partially blocked Ang II-induced pregnenolone synthesis at 3 μM (30% inhibition of Ang II response), it dramatically decreased K^+-induced pregnenolone production at the same concentration (70% inhibition of K^+ response, IC_{50} 0.9 μM). When CEH was immunoprecipitated from glomerulosa cells coincubated

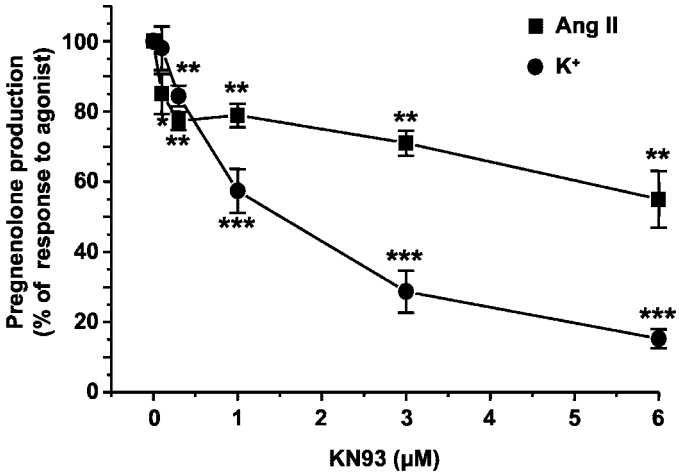

FIG. 7. Effect of KN93 on agonist-stimulated pregnenolone production. Glomerulosa cells were preincubated in the presence or absence of KN93 (0.3–6 μM) for 30 min before stimulation with 10 nM Ang II or 12 mM K^+ for 2 h. Pregnenolone content of the incubation medium was determined by RIA and normalized to the cellular protein content. In a representative experiment, pregnenolone production was 0.3, 9.2, and 4.1 pmol/min·mg protein for controls and Ang II- and K^+-stimulated cells, respectively. Data points represent the mean ± SEM of duplicate determinations from six independent experiments, expressed as percent of pregnenolone production measured with Ang II or K^+ alone (**, ***, Significantly different from Ang II or K^+ alone with $P < 0.01$ and $P < 0.0001$, respectively, n = 6).

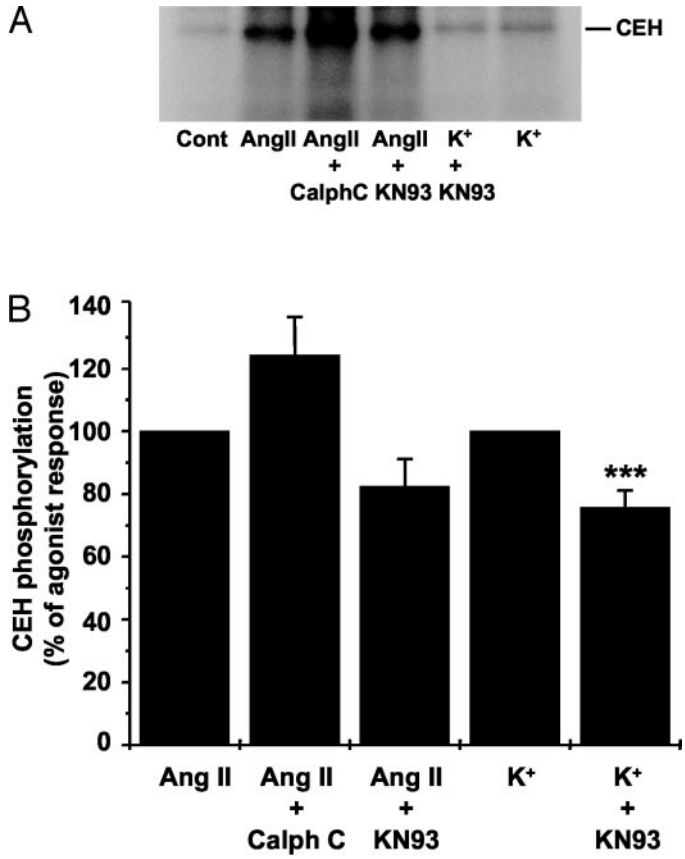

FIG. 8. Neither CaMKII nor PKC are involved in Ang II-induced phosphorylation of CEH. Glomerulosa cells were pretreated with 3 μM KN93 or 1 μM calphostin C (Calph C) for 30 min before stimulation with 10 nM Ang II or 12 mM K^+. Phosphorylated CEH was immunoprecipitated as described in *Materials and Methods* and analyzed by SDS-PAGE and autoradiography. A, Shown is an autoradiogram representative of four to five independent experiments. B, Densitometric scanning analysis of phosphorylated CEH (n = 5 for Ang II ± KN93 and Ang II ± calphostin C; n = 4 for K^+ ± KN93, ***, Significantly different from response to K^+, $P < 0.0001$).

with 3 μM KN93 and Ang II or K^+ (Fig. 8A), the inhibitor did not affect significantly Ang II-induced CEH phosphorylation. Indeed, densitometric analysis of five independent experiments indicated that 82 ± 9% of the Ang II-induced phosphorylation was still detectable in the presence of KN93 (Fig. 8B). K^+-induced CEH phosphorylation was partially but significantly inhibited in the presence of KN93, to 75 ± 4% of controls (n = 4, $P < 0.001$, Fig. 8, A and B).

Because one of the well-characterized signaling events triggered by Ang II is activation of PKC, we examined the possible involvement of this kinase in Ang II-mediated CEH phosphorylation using calphostin C, a potent selective inhibitor of PKC. As shown in Fig. 8, A and B, calphostin C (1 μM) had either no effect or even potentiated phosphorylation of the enzyme by Ang II (124 ± 12% of Ang II response, n = 5).

Involvement of MAPK in Ang II-induced phosphorylation of CEH

It has been clearly demonstrated that Ang II stimulates the MAPK cascade in the adrenal gland. The p42/p44 MAPK is involved in the growth-promoting effects of Ang II on both fasciculata and glomerulosa cells of the adrenal cortex (37–40). However, the role of MAPK in the differentiated function of adrenocortical cells, *i.e.* steroid biosynthesis, has been poorly investigated due to the fact that, until recently, specific inhibitors for kinases of this family were lacking. A previous study by Tian *et al.* (38) reported that Ang II triggers a rapid and transient activation of p42 MAPK in bovine adrenal glomerulosa cells, as measured by changes in its electrophoretic mobility, which reflects its dual phosphorylation on threonine and tyrosine residues. Here we investigated the activation of both p42 and p44 MAPK by Ang II, using a specific antibody that recognizes the phosphorylated forms of both kinases. The kinetics of MAPK activation by Ang II show maximal phosphorylation between 5 and 10 min after treatment (873% and 742% of basal values for p42 and p44 MAPK, respectively, n = 3), decreasing to half-maximal phosphorylation by 60 min and remaining higher than the basal level for up to 2 h (Fig. 9, A and D). The membrane was stripped and reprobed with an antibody recognizing total levels of p42/p44 MAPK to check for equal loading of protein into each lane (Fig. 9B). Densitometric scanning analysis of this representative experiment yielded a 2:1 ratio for p42/p44 MAPK, indicating that p42 MAPK is the isoform preferentially expressed in bovine glomerulosa cells.

To examine whether MAPK could be involved in the early steps of aldosterone biosynthesis, we used recently developed selective inhibitors of the MAPK cascade, PD098059 for the p42/p44 MAPK kinase and SB203580 as for the stress-activated p38 MAPK. PD098059 (50 μM), which prevented p42/p44 MAPK phosphorylation elicited by Ang II (Fig. 9C), also decreased Ang II-stimulated pregnenolone production

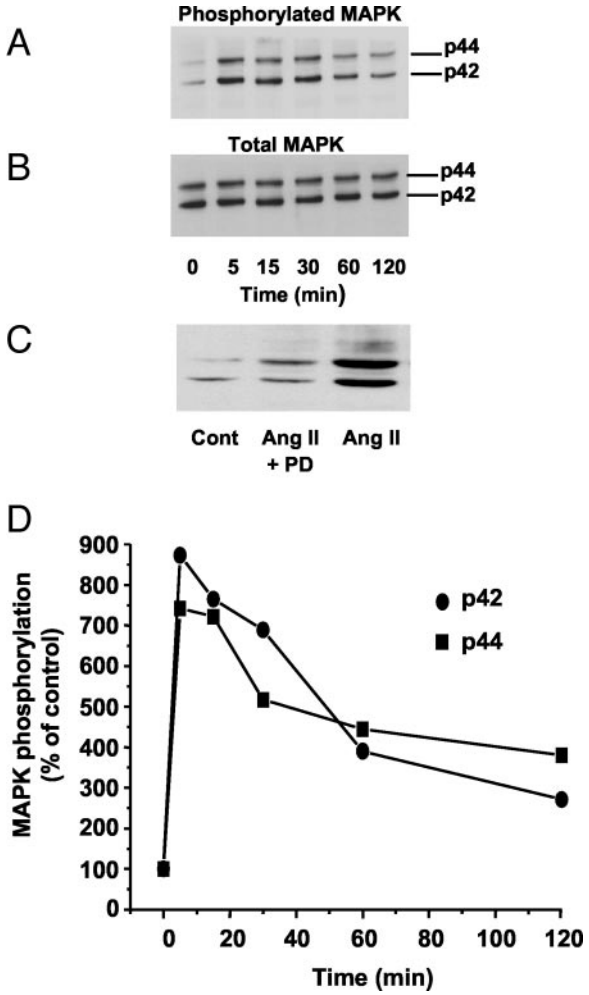

FIG. 9. Time course of Ang II-induced phosphorylation of MAPK. Glomerulosa cells were exposed to 10 nM Ang II for the indicated times. Cells lysates were prepared as described in *Materials and Methods* and subjected to immunoblotting (10 μg protein/lane). A, Immunoblotting with an antibody recognizing specifically the phosphorylated forms of p42/p44 MAPK. B, The immunoblot in A was stripped and reprobed with an antibody recognizing total levels of p42/p44 MAPK to check for equal loading of protein in each lane. C, Effect of PD098059 on Ang II-induced p42/p44 MAPK phosphorylation. D, Densitometric scanning analysis of the immunoblot shown in A. The phosphorylation level of p42/p44 MAPK in Ang II-treated cells is expressed as a percentage of that detected in control cells. Data are representative of three independent experiments.

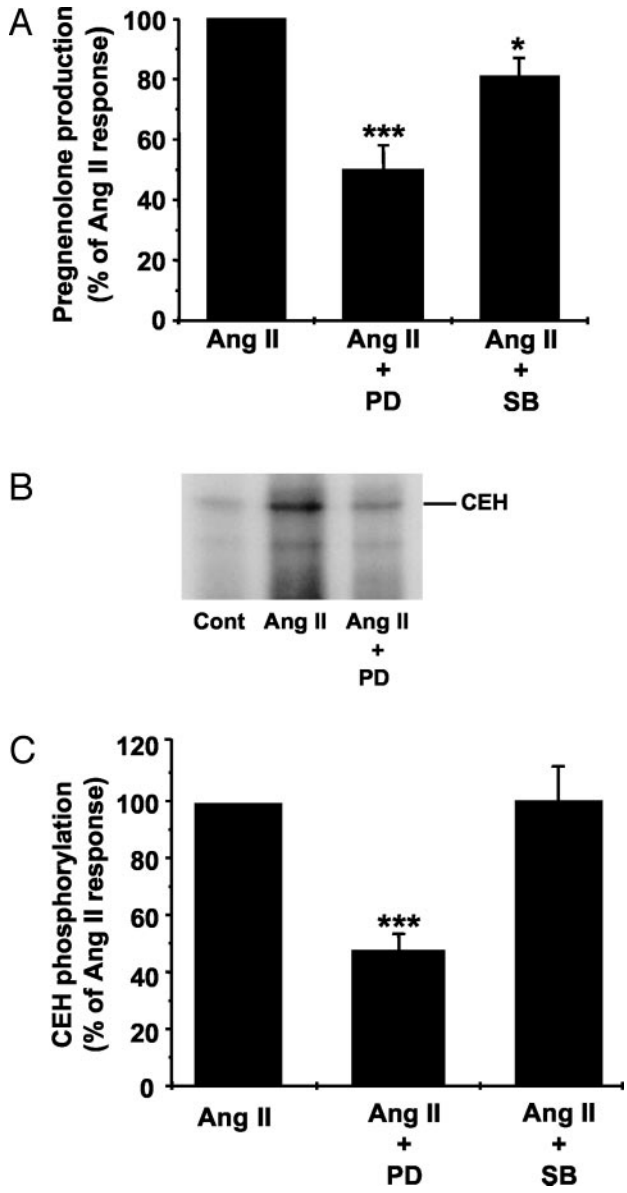

FIG. 10. p42/p44 MAPK is involved in Ang II-induced phosphorylation of CEH. Glomerulosa cells were exposed to 50 μM PD098059 (PD) or 10 μM SB203580 (SB) for 30 min before stimulation with 10 nM Ang II for 2 h. A, Effect of PD098059 and SB203580 on pregnenolone production. Data are the mean ± SEM of four independent experiments assayed in duplicate (*, ***, Significantly different from response to Ang II alone, with $P < 0.05$ and $P < 0.001$, respectively). B, SDS-PAGE analysis and autoradiography of CEH immunoprecipitated from glomerulosa cells stimulated with Ang II in the presence or absence of PD098059. C, Densitometric scanning analysis of phosphorylated CEH from four and three independent experiments for Ang II ± PD098059 and Ang II ± SB203580, respectively. ***, Significantly different from the response to Ang II, $P < 0.001$.

to 50 ± 8.2% of controls with Ang II alone (n = 4, $P < 0.01$), whereas SB203580 (10 μM) had only a minor effect (Fig. 10A). We next tested the effect of PD098059 on Ang II-induced CEH phosphorylation (Fig. 10, B and C). Cotreatment of glomerulosa cells with Ang II and PD098059 decreased Ang II-induced CEH phosphorylation to 47.4 ± 6% (n = 4, $P < 0.001$). By contrast, SB203580 had no significant effect on CEH phosphorylation by Ang II (98.9 ± 12%, n = 3). These results indicate that the p42/44 MAPK is a significant mediator of Ang II-induced phosphorylation of CEH and thereby contributes to the regulation of cholesterol delivery to mitochondria.

Discussion

Trophic hormone stimulation of steroidogenic cells results in a prompt conversion of cholesterol esters stored in cytoplasmic lipid droplets into free cholesterol, the precursor of all steroid hormones. This process involves CEH, a key enzyme in determining cellular levels of free and esterified cholesterol. Pioneering work by Behrman and Armstrong

(41) in the late 1960s has shown, for example, that LH stimulates CEH in luteinized rat ovaries, thus enhancing intracellular transfer of cholesterol to the mitochondria. It is well established that CEH activity is modulated through reversible phosphorylation of the enzyme by the cAMP-dependent protein kinase (PKA). In the present study, we characterized CEH activity and regulation in steroidogenic cells that rely on the calcium messenger system for their activation, the adrenal glomerulosa cells. We demonstrated that 1) Ang II and K^+, the major physiological stimuli of aldosterone biosynthesis in glomerulosa cells, increase CEH activity; 2) both agonists increase CEH phosphorylation; and 3) the MAPK signaling pathway mediates a major part of Ang II action on CEH.

Enzymatic characterization of CEH activity in bovine glomerulosa cells yielded kinetic constants similar to those reported by others in adrenal cortex tissue of various species (4, 8, 42) as well as in mouse Y1 adrenal tumor cells (30). It is worth mentioning that CEH-specific activity was higher in fresh adrenal glomerulosa tissue, suggesting either that an up-regulating factor is removed during cell culture or, more likely, that stress induces a marked increase in adrenal CEH activity, as reported by others (1, 2, 9). This observation was confirmed by Western blot analysis of CEH, which revealed that enzyme expression was higher in fresh isolated tissue, compared with that of cultured cells (data not shown).

Ang II and K^+ promote cholesterol supply for steroidogenesis by increasing CEH enzymatic activity. These increases are similar to those reported in the adrenal gland of rat and guinea pig subjected to ether stress or ACTH injection (approximately a 2-fold increase over controls) (5, 6, 8) and in adrenal tumor cell monolayers treated with ACTH or dbcAMP (1.5-fold over controls) (30). Remarkably, this 1.5-fold increase in CEH activity above basal is rather modest when compared with the up to 15- and 30-fold stimulation of steroidogenesis observed in intact cells stimulated with K^+ and Ang II, respectively. This discrepancy is consistent with the concept that the supply of free cholesterol via CEH-mediated hydrolysis of cholesteryl esters is not rate limiting for steroidogenesis under the current conditions. Conversely, it has been suggested that the inability to detect large increases in CEH activity *in vitro* was due to a large interface of the lipid substrate, available in excess, with the enzyme. This results in a relatively high basal enzyme activity and consequently reduces the fold induction of cholesterol ester hydrolysis rate measured in agonist-stimulated cells (43). Furthermore, evidence is accumulating that activation of lipolysis is associated with the translocation of HSL from the cytosolic compartment to the triglyceride droplet (44, 45). A similar mechanism might exist for CEH regulation and could explain, for example, the observed dissociation between CEH phosphorylation and activity. Finally, our observation that ionomycin, which evokes a diffuse rise in cytosolic Ca^{2+} concentration, was ineffective in increasing CEH activity supports the hypothesis that CEH activation in glomerulosa cells requires localized Ca^{2+} rises (46).

We used antibodies generated against HSL, the homolog of CEH in adipose tissue, to explore CEH regulation in glomerulosa cells. Hereafter we use HSL to refer to the adipose tissue enzyme, whereas CEH refers to the enzyme found in the adrenal cortex. Previous studies have shown that purified CEH and HSL are the same enzyme based on their substrate specificities; sensitivity to inhibition by sodium and diisopropyl fluorophosphate; subunit molecular weight; ability to be phosphorylated by PKA; effect of phosphorylation on enzyme activity; and, finally, polypeptide degradation pattern following limited proteolysis (12–15). These results were corroborated by the observation that CEH activity was markedly decreased in Chinese hamster ovary cells transfected with antisense HSL cDNA and that HSL mRNA in adipose and adrenal tissues exhibits the same molecular weight (47, 48). More recently, it has been demonstrated that neutral CEH activity was completely abolished in HSL null mice (50). In addition, Li *et al.* (51) provided evidence showing that HSL-deficient mice exhibit lipid accumulation in the adrenal cortex and impaired steroidogenic response to hormone stimulation. Thus, HSL appears to be responsible for most, if not all, of adrenal CEH activity. In agreement with previous studies conducted in steroidogenic and adipose tissues (11–16, 25, 46, 52, 53), we observed an apparent molecular mass of 83 kDa for CEH in glomerulosa cells. Whether the 65-kDa protein also detected in these cells represents an isoform of CEH or whether it is an immunologically related but functionally irrelevant protein remains to be determined. Neither Ang II nor K^+ or forskolin affected CEH protein content in glomerulosa cells within 2 h of stimulation, in agreement with previous studies demonstrating that ACTH-induced cholesterol esters hydrolysis was not affected by the protein synthesis inhibitor cycloheximide (54).

The Ang II- and K^+-induced increase in CEH activity is accompanied by an increase in CEH phosphorylation state, the effect of Ang II being more marked than that of K^+. The 172-kDa form of CEH immunoprecipitated from glomerulosa cell extracts is most likely a dimer of the 83-kDa enzyme. Indeed, the phosphorylated 172-kDa doublet is displaced by increasing concentrations of purified HSL fusion protein. Moreover, it has been recently shown that HSL functions as a dimer in adipose tissue and that phosphorylation appears to be important in enabling dimerization to occur (52). Although it is theoretically possible that the 172-kDa doublet represents CEH bound to another phosphorylated protein of similar size that coprecipitates with anti-HSL antibodies, this possibility seems, however, unlikely because a similarly sized dimer is seen when recombinant HSL is produced in a baculovirus system (52). It is also worth mentioning here that cholate-type detergents induce the formation of CEH dimers in SDS gels, presumably via "strong intrachain hydrophobic interactions that are not readily disrupted by SDS" (53).

The physiological relevance of phosphorylation as a regulatory mechanism for CEH in adrenal glomerulosa cells is supported by the observation that the enzyme is indeed phosphorylated in intact cells and that the degree of phosphorylation correlates with the rate of cholesterol ester hydrolysis under Ang II or K^+ challenge. Interestingly, we found that Ang II induced a sustained increase in CEH phosphorylation, indicating that mobilization of cholesterol esters is maintained as long as the hormone is present. The kinetics of the Ang II-induced phosphorylation of CEH in intact cells is reasonably comparable with that reported for PKA-

induced phosphorylation (16), considering that the latter study has been performed in a purified system. Taken together, these results indicate that the Ca^{2+} and cAMP messenger systems converge to modulate CEH phosphorylation, although the amino acid residue(s) phosphorylated after activation of the Ca^{2+} signaling pathway remain(s) to be identified. Indeed, bovine- and rat-purified HSLs can be phosphorylated on two distinct serine residues, termed the regulatory and basal sites. PKA has been shown in most studies to phosphorylate HSL at a single regulatory site identified as Ser-563 in rat HSL (55) or site 1 in bovine HSL (56). Another report identified two additional phosphorylated residues in rat HSL, Ser-659 and Ser-660 (57). In bovine adrenal cortex, proteolytic digestion of CEH, followed by peptide mapping indicated that CEH was phosphorylated by PKA on a single serine residue (16). Phosphorylation of the basal site in HSL (Ser-565 and site 2 in rat and bovine HSL, respectively), shown to be catalyzed by AMP-activated kinase (58) or by CaMKII (55), impairs phosphorylation of the regulatory site by PKA, thus inhibiting cholesterol ester hydrolysis by the enzyme.

The MAPK signaling pathway mediates a significant part (approximately 50%) of Ang II action on CEH. Whereas the CaMKII inhibitor, KN93, did not significantly alter Ang II-mediated CEH phosphorylation, we cannot exclude that a different isoform of the CAMK family or a downstream kinase might mediate the other 50% of CEH phosphorylation on the regulatory or another site. In fact, we observed that removal of extracellular Ca^{2+} dramatically reduced the effect of Ang II on CEH phosphorylation by 60%, indicating an important role for Ca^{2+} in Ang II-induced phosphorylation of CEH (data not shown). In this respect, it is worth mentioning that CaMKI has been recently reported to be the major isoform of CaMK expressed in adrenal cortex and the human adrenal H295 cell line, followed by CaMKIV, whereas CaMKII was undetectable (59). The modest inhibition of Ang II-stimulated pregnenolone production elicited by KN93 suggests that the target of CaMK may lay downstream of CEH activation and upstream of pregnenolone production, possibly at the level of StAR-mediated intramitochondrial cholesterol transfer. We have indeed recently shown that changes in intracellular calcium concentration will lead to increased expression of the *StAR* gene (27) and mitochondrial importation of the StAR protein (24).

By contrast, K^+-induced pregnenolone production was severely impaired in the presence of KN93, indicating that CaMK activation is a key step in K^+-stimulated pregnenolone synthesis. Similar results have been obtained with human H295 adrenocarcinoma cells, in which aldosterone synthesis was fully inhibited by KN93 (36). However, KN93 inhibited only partially K^+-induced CEH phosphorylation, thus making a major contribution of CaMKII in the K^+-induced increase in CEH activity unlikely.

In glomerulosa cells, the role of PKC in aldosterone synthesis is a matter of debate. Although various reports including work from our own group (60, 61) have shown that activation of PKC by the phorbol ester PMA leads either to no effect or inhibition of aldosterone production in Ang II-stimulated cells, others (62) have claimed that PKC is positively involved in this process. Our data strongly suggest that PKC is not involved in Ang II-induced phosphorylation of CEH. In fact, inhibition of PKC even resulted in a potentiation of Ang II-mediated phosphorylation of CEH, thus confirming our previous studies on the negative role of PKC in aldosterone synthesis.

The adrenal cortex is a major trophic target for Ang II. Mitogenic effects of the hormone have been recognized in both the zona fasciculata/reticularis and the zona glomerulosa (37–40). In bovine glomerulosa cells, the growth-promoting effect of Ang II is mediated by activation of the MAPK signaling pathway via angiotensin II type 1 receptors coupled to G_q (38). Ang II-induced MAPK activation appears to involve at least two pathways: a major PKC-dependent pathway and a minor PKC-independent pathway shown to operate via the ras/raf-1 cascade (38–40). However, the possible involvement of MAPK in the acute stimulation of steroidogenesis has been poorly investigated. In a recent study, Côté *et al.* (39) reported that the MAPK inhibitor PD098059 had no effect on aldosterone production in rat adrenal glomerulosa cells. In our hands, PD098059 markedly inhibited Ang II-stimulated pregnenolone production in bovine glomerulosa cells, suggesting that specific target(s) for MAPK are located in the early steps of steroidogenesis. Indeed, the Ang II-induced increase in CEH phosphorylation was markedly decreased in the presence of PD098059, thus clearly showing a role for MAPK in the control of adrenal CEH. Similarly, it has been recently reported that β-adrenergic agonist-induced lipolysis in adipocytes is partially blocked by PD098059 and that HSL is phosphorylated *in vitro* and *in vivo* by activated MAPK (63), a finding demonstrating that the MAPK pathway is at least in part involved in the regulation of HSL activity by β-adrenergic agonists. Furthermore, review of the HSL amino acid sequence indicated regions consistent with consensus MAPK phosphorylation sites (64). Recently Ser^{600} has be shown as the site that is phosphorylated by activated MAPK in HSL (63). This phosphorylated site is required for activated MAPK to increase HSL activity in adipocytes. Because PKC exerts a negative feedback on Ang II-induced phosphorylation of CEH, it is unlikely that the PKC-dependent activation of MAPK cascade mediates the phosphorylation of CEH. Whether the ras/raf-1 signaling pathway is involved in CEH phosphorylation in adrenal glomerulosa cells remains to be determined.

In conclusion, we demonstrated that, in addition to its previously reported effects on rate-limiting steps of aldosterone biosynthesis, Ang II phosphorylates and activates CEH, a key enzyme in providing cholesterol for steroidogenesis. Importantly, the MAPK cascade plays a significant part in this effect of Ang II, thus demonstrating that, in addition to its involvement in long-term trophic responses, this pathway also participates in the short-term steroidogenic response.

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Address all correspondence and requests for reprints to: Professor Alessandro Capponi, Division of Endocrinology, Diabetology and Nutrition, University Hospital, 24, Micheli-du-Crest, CH-1211 Geneva 14, Switzerland. E-mail: alessandro.capponi@medecine.unige.ch.

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