Regulation of aldosterone production from zona glomerulosa cells by ANG II and cAMP: evidence for PKA-independent activation of CaMK by cAMP

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Gambaryan, Stepan, Elke Butt, Piet Tas, Albert Smolenski, Bruno Allolio, and Ulrich Walter. Regulation of aldosterone production from zona glomerulosa cells by ANG II and cAMP: evidence for PKA-independent activation of CaMK by cAMP. Am J Physiol Endocrinol Metab 290: E423–E433, 2006.—Aldosterone production in zona glomerulosa (ZG) cells of adrenal glands is regulated by various extracellular stimuli (K+; ANG II; ACTH) that all converge on two major intracellular signaling pathways: an increase in cAMP production and calcium (Ca2+) mobilization. However, molecular events downstream of the increase in intracellular cAMP and Ca2+ content are controversial and far from being completely resolved. Here, we found that Ca2+/calmodulin-dependent protein kinases (CaMks) play a predominant role in the regulation of aldosterone production stimulated by ANG II, ACTH, and cAMP. The specific CaMK inhibitor KN93 strongly reduced ANG II-, ACTH-, and cAMP-stimulated aldosterone production. In in vitro kinase assays and intact cells, we could show that cAMP-induced activation of CaMk, using the adenylate cyclase activator forskolin or the cAMP-agonist Sp-5,6-DCl-cBIMPS (cBIMPS), was not mediated by PKA. Activation of the recently identified cAMP target protein Epac (exchange protein directly activated by cAMP) by 8-pCPT-2’-O-Me-cAMP had no effect on CaMk activity and aldosterone production. Furthermore, we provide evidence that cAMP effects in ZG cells do not involve Ca2+ or MAPK signaling. Our results suggest that ZG cells, in addition to PKA and Epac/Rap proteins, contain other as yet unidentified cAMP mediator(s) involved in regulating CaMk activity and aldosterone secretion.

angiotension II; adenosine 3’,5’-cyclic monophosphate; adenosine 3’,5’-cyclic monophosphate-dependent protein kinase; Ca2+/calmodulin-dependent kinase

ALL STEROID HORMONES, INCLUDING ALDOSTERONE, are synthesized from a common precursor, cholesterol. Stimulation of steroid-producing cells results in prompt mobilization of cholesterol esters from intracellular lipid droplets and their enzymatic hydrolysis to free cholesterol by cholesterol ester hydrolase (CEH) (8). Free cholesterol is then transported to the outer mitochondrial membrane (28, 29, 50, 57), where it is transferred to the side-chain cleavage enzyme system that is localized at the inner mitochondrial membranes. The latter step is the rate-limiting step in steroidogenesis and depends on the activity and phosphorylation of the steroidogenic acute regulatory protein (StAR) (49).

The steroid hormone aldosterone produced in adrenal zona glomerulosa (ZG) cells is a major regulator of intravascular volume and blood pressure. Many hormonal and paracrine factors are involved in the regulation of aldosterone production; however, under physiological conditions, the most important ones are adrenocorticotropin (ACTH), angiotensin II (ANG II), and extracellular K+ (19, 46).

The main action of ACTH in ZG cells is connected with the activation of transmembrane G×α-coupled ACTH receptors and the generation of cAMP as a second messenger (11, 37). Until the recent discovery of a new family of cAMP-binding proteins [exchange proteins directly activated by cAMP (Epacs)] that can directly activate small GTPases Rap1 and Rap2 (12), cAMP signaling was mainly linked to the activation of cAMP-dependent kinase protein (PKA) and subsequent phosphorylation of target proteins. Hydrolysis of cholesterol esters to free cholesterol by CEH and activation of StAR, which are both critical steps in steroidogenesis, are regulated by PKA phosphorylation. Phosphorylation of CEH by PKA markedly decreases cholesterol ester levels and increases CEH activity (9, 22). Phosphorylation of StAR by PKA at Ser65 and Ser194 increases the delivery of sterol substrate to the inner mitochondrial membrane and, in part, accounts for the acute effect on the activation of steroid production (2). Although ACTH in ZG cells acts primarily through cAMP, it also can stimulate calcium (Ca2+) influx by activation of L-type Ca2+ channels (14, 18, 41). However, an increase of intracellular Ca2+ ([Ca2+]i) was detected at very high (100 nM) ACTH concentrations and was abolished by the addition of PKA inhibitor H-89 (18).

Despite the fact that one of the Epacs, namely Epac2, is predominantly expressed in the adrenal gland (4) and may be localized in the mitochondria (23), where steroidogenesis takes place and Rap1 and Rap2 are expressed in ZG cells at relatively high levels (see RESULTS), the involvement of this signaling cascade in ACTH-dependent steroidogenesis in ZG cells remains unknown.

ANG II stimulates aldosterone production by activating the ANG II type 1 (AT1) receptor, the predominant receptor isoform expressed in rat and bovine ZG cells (3). Stimulation of the AT1 receptor initiates a cascade of signaling events that includes activation of phosphoinositide-specific phospholipase C and hydrolysis of phosphatidylinositol 4,5-bisphosphate, yielding soluble inositol 1,4,5-trisphosphate (IP3), and diacylglycerol (DAG). Although DAG activates protein kinase C (PKC), IP3 induces the release of Ca2+ from intracellular stores, followed by the activation of store-operated Ca2+ channels. ANG II also activates both T- and L-type Ca2+ channels.
by inducing cell depolarization through the inhibition of K+ channels (6, 38). One of the major signaling systems that is activated by elevated intracellular Ca2+ includes Ca2+/calmodulin-dependent protein kinases (CaMKs) that transduce elevated Ca2+ signals to a number of target proteins.

Multifunctional CaMKs (CaMKI, -II, -IV, and CaMK kinase (CaMKK)) play important roles in controlling a variety of cellular functions in response to an increase of intracellular Ca2+ (28, 30, 45, 46). CaMKs are involved in the regulation of aldosterone production stimulated by ANG II and K+ (10, 15, 16, 40, 42). In earlier papers, CaMKII was proposed to be the enzyme responsible for aldosterone production stimulated by ANG II. It was shown later that in human NC1-H295R cells, CaMKI plays a predominant role in the activation of aldosterone synthase expression (10).

Here, we demonstrate that rat and bovine ZG cells express all CaMKs [CaMKI, -II, -IV, and CaMK kinase (CaMKK)] at approximately similar levels. We found that CaMKs play a predominant role in regulation of aldosterone production stimulated with ANG II, as well as with cAMP. ANG II-stimulated aldosterone production was significantly inhibited by both CaMKK-specific inhibitor and CaMKII inhibitory peptide, whereas ACTH-stimulated aldosterone production was inhibited by only a CaMKK-specific inhibitor. In addition, we show that cAMP-stimulated CaMK activity is independent from PKA and Epac/Rap1 signaling systems by using in vitro kinase assays and experiments with intact cells. These data suggest that ZG cells may have a new cAMP mediator involved in the regulation of CaMK activity.

MATERIALS AND METHODS

Preparation of ZG cells. Rat (male Sprague-Dawley, 200–300 g) and bovine (adrenal glands were obtained from a local slaughterhouse) adrenal ZG cells attached to the adrenal capsule were digested with collagenase and mechanically disaggregated as described (18–21). Isolated cells were suspended in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Most of the experiments, except pull-down and kinase assays that were done only on bovine ZG cells, were made on both rat and bovine ZG cells, and there were no significant differences between these two models. For the recording of [Ca2+]i, cells were plated on glass coverslips (40 mm diameter) at a density of 100,000 cells/ml and after 2 days were used for [Ca2+]i, recording. For experiments on aldosterone production and Western blotting, the cells were plated into six-well plates at a density of 10–12 × 10⁶ cells/dish. For CaMK in vitro kinase assays and Rap1 and Rap2 pull-down assays, the cells were plated into 15-cm dishes at a density of 10–12 × 10⁶ cells/dish. All experiments were done with confluent cells (after 3 days in culture). The medium was replaced by serum-free DMEM for 12 h (for aldosterone production experiments, DMEM was supplemented with 0.5% FCS). For aldosterone production experiments, cells were incubated for 1 h with different combinations of substances to be tested: ANG II, KN93, KN92, H-89 (all from Calbiochem, Sigma, Deisenhofen, Germany), cell-permeable cAMP analogs Sp-5,6-DCl-cBIMPS and 8-pCPT-2′-O-Me-cAMP (BioLog, Bremen, Germany), and ST-609 (Biotrend, Cologne, Germany). CaMKII inhibitory peptide 27-mer CaMKII Ntide (CNT) is derived from CaMKII and selectively inhibits CaMKII activity (7). To generate cell-permeable peptide, an antennapedia sequence (RQIKIWFQNRRMKWKK) CaMKII inhibitory peptide (antiCNT) was placed NH2-terminal to CNT (antiCNT) as described (17). The antiCNT was synthesized by GenScript (Piscataway, NJ). Separate experiments were done in DMEM without Ca2+. After incubation with substances, the medium was collected for aldosterone RIA. For Western blot analysis, CaMK in vitro kinase assays, and pull-down assays, cells were preincubated with inhibitors for 10 min and then stimulated with ANG II or forskolin for 5 min and harvested in lysis buffer or SDS gel loading buffer.

Intracellular Ca2+ measurements. For the recording of intracellular Ca2+ gradients, cells attached to the coverslip were loaded with 5 µM of the Ca2+ indicator fluo-3 AM (Molecular Probes, Gottingen, Germany) for 30 min at 37°C in a buffer containing 25 mM HEPES, pH 7.0, 0.4 mM MgCl2, 5 mM KCl, 150 mM NaCl, 2 mM CaCl2, and 25 mM d-glucose (buffer C). The coverslips with the cells were then mounted in a gas-tight, temperature-controlled perfusion chamber (Biotech, Butler, PA) and perfused with buffer C for 5 min. After this resting time, cells were perfused with inhibitors or directly with forskolin or ANG II. Changes in fluorescence, corresponding to changes in [Ca2+], were monitored with a confocal laser-scanning microscope (MRC1024; Bio-Rad, Munich, Germany) equipped with an argon-krypton laser. Fluo-3 excitation was achieved with the 488-nm laser line. The fluorescence recordings of individual cells were monitored with the LaserSharp Acquisition software (Bio-Rad).

Western blot analysis. After stimulation, cultured ZG cells were washed with PBS, and cells were harvested in SDS gel loading buffer. Samples were analyzed by Western blot using the following antibodies: rabbit polyclonal anti-phospho/rabbit polyclonal anti-phospho (Thr180/Tyr182)-p38 MAPK, anti-phospho (Thr286)-MAPKAP2, anti-phospho (Ser197)-Rap1, anti-p38, anti-ERK (all from Cell Signaling, Frankfurt/Main, Germany), anti-phospho (Thr286/Tyr204)-ERK, (Nanotools, Frieburg, Germany). The following antibodies were used for the demonstration of CaMK expression in rat and bovine ZG cells: against CaMKI goat monoclonal (C-19; Santa Cruz Biotechnol, Santa Cruz, CA), against CaMKII, CaMKIV, and CaMKK, mouse monoclonal (BD Biosciences, Heidelberg, Germany). PKA activity was evaluated by the phosphorylation of the well-known PKA substrate vasodilator-stimulated phosphoprotein (VASP) detected by monoclonal anti-phospho (Ser²³⁹)-VASP mouse antibody (44a). Equal loading was controlled by Ponceau S staining of the membrane and incubation of the stripped membranes with corresponding non-phospho antibodies. For visualization of the signal, goat anti-rabbit or rabbit anti-goat IgG conjugated with horseradish peroxidase was used as a secondary antibody, followed by enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Freiburg, Germany).

Kinase assay. For the CaMK activity assay, cells were collected in lysis buffer (10 mM Tris–HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 0.1 mM IBMX, 0.5 mM PMSF, 10 mg/ml leupeptin and pepstatin, 10 mM Calyculin A) and then disrupted by passing them five times through a 29-gauge needle. The protein concentration was determined using a microbichinonic acid assay with bovine serum albumin as the standard. CaMK activity was measured using a synthetic peptide (autocamtide-3; KKLHRGETVDAL, Calbiochem) as a substrate. Phosphorylation reactions were initiated by adding 10 µl of cell homogenate (10 µg protein) to a 20-µl reaction mixture, resulting in the following final concentrations: 10 mM HEPES (pH 7.4), 0.5 mM DTT, 3 mM EGTA, 5 mM MgCl2, 10 µM autocomtide-3, 50 µM ATP, 3 µCi of [γ-32P]ATP (3,000 Ci/mmol). After a 2-min incubation at 30°C, the reactions were terminated by spotting 15 µl of the reaction mixture onto P-81 phosphocellulose filters. The filters were washed three times (10 min) in 75 mM phosphoric acid, rinsed with ethanol, and air dried. The radioactivity on the phosphorylated autocamtide-3 was quantitated in a scintillation counter by the Cerenkov method.

Pull-down assays. Activated RhoA, Rac, Cdc42, Rap1, and Rap2 were determined by pull-down assays with corresponding binding proteins (Rhotekin-Rho-binding domain for RhoA, Rac-GTP-binding domain of PAK for Rac and Cdc42, and Rap-binding domain of RalGDS for Rap1 and Rap2). All binding domains were expressed as GST fusion proteins purified with glutathione-agarose beads (Amer-
with 5 from ZG cells have not yet been described. We determined that PKA-independent effects of cAMP on aldosterone production mediate the StAR protein and activate StAR gene expression (50).

Results of forskolin, ANG II, or 8-pCPT-2′-O-Me-cAMP (for Rap1 and Rap2 activation) for 5 min and then washed twice with ice-cold PBS and harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 10% glycerol) containing protease inhibitors (Complete; Roche Applied Science, Mannheim, Germany). Cells were then disrupted by passing them five times through a 29-gauge needle, and the lysate was centrifuged for 15 min at 14,000 rpm at 4°C. An aliquot (50 μl) of the supernatant was used to estimate the total amount of GTPases. The remaining supernatant was mixed with the corresponding glutathione-agarose beads and incubated for 45 min at 4°C. The samples were then centrifuged at 320 g, and the beads were washed three times with lysis buffer. Finally, the pellet beads were resuspended in SDS gel-loading buffer and analyzed by 12% SDS-PAGE using anti-RhoA (Santa Cruz Biotechnology), anti-Rac and anti-Cdc42 (Upstate-Biomed), and anti-Rap1 and -Rap2 (Transduction Laboratories) monoclonal antibodies.

Aldosterone RIA. Aldosterone concentration in the culture medium was determined by RIA using a commercially available aldosterone kit (DPC Biermann, Bad Nauheim, Germany).

Statistical analysis. The data shown were pooled from at least three experiments. Values are expressed as means ± SE. Differences among groups were analyzed by Student’s t-test, and P < 0.05 was considered statistically significant.

Results

Inhibition of forskolin, cBIMPS, and ACTH-stimulated aldosterone production by CaMK inhibitors is independent of PKA activity. cAMP, which is elevated by the stimulation of ZG cells with ACTH or forskolin, is one of the most potent activators of aldosterone production. It is generally accepted that in ZG cells cAMP stimulation of aldosterone production is mediated by activation of PKA, which can directly phosphorylate the StAR protein and activate StAR gene expression (50). PKA-independent effects of cAMP on aldosterone production from ZG cells have not yet been described. We determined that the preincubation of rat (data not shown) and bovine ZG cells with 5 μM of the specific PKA inhibitor H-89 dramatically inhibited PKA activity [assessed by VASP phosphorylation, phosphorylated (P)-VASP Westerns; Fig. 1], although the aldosterone production decreased by no more than 30%. By contrast, the specific CaMK inhibitor KN93 (5 μM) totally inhibited aldosterone production without any effect on PKA activity (Fig. 1A). KN92, a structurally similar analog of KN93 that does not affect CaMK enzymatic activity, had no effect on aldosterone production and VASP phosphorylation stimulated by forskolin (Fig. 1A). To exclude possible nonspecific stimulation of CaMK by forskolin, similar experiments were performed with the cell-permeable cAMP analog cBIMPS (Fig. 1B) and ACTH (data not shown). There was no significant difference among forskolin, cBIMPS, and ACTH effect on aldosterone production and VASP phosphorylation (compare Fig. 1A and 1B).

Elevation of cAMP by forskolin, ACTH, and cAMP analogs has no effect on [Ca2⁺]. Classically, activation of CaMK requires elevation of [Ca2⁺]; therefore, we determined whether the elevation of cAMP by forskolin or 10 nM ACTH and the cAMP analog cBIMPS would increase [Ca2⁺] in ZG cells. In rat ZG cells, forskolin (5 μM) and ACTH (10 nM) elevated the cAMP concentration within 5 min >20-fold and 5-fold, respectively (data not shown), but this increase had no effect on [Ca2⁺]. Both forskolin and ACTH also did not change ANG II-induced elevation of [Ca2⁺], (Fig. 2, A and B). ACTH at a high concentration (100 nM) can increase [Ca2⁺] by PKA-dependent stimulation of L-type Ca2⁺ channels (18). However, in our experiments, ACTH, forskolin, or cBIMPS did not activate L-type Ca2⁺ channels, which can be activated by the addition of 50 mM KCl and blocked with 1 μM of specific L-type Ca2⁺ channel inhibitor nifedipine (Fig. 2C).
A CaMK inhibitor inhibits ANG II-induced aldosterone production without inhibiting ANG II increase in [Ca\(^{2+}\)]. Because inhibition of Ca\(^{2+}\) channels and the subsequent decrease in [Ca\(^{2+}\)], also blocks ANG II-induced aldosterone production (6, 38), we further tested the ability of KN93 to inhibit the rise in [Ca\(^{2+}\)] induced by ANG II. ANG II-induced aldosterone production was blocked by the specific CaMK inhibitor KN93, whereas KN92 and PKA inhibitor H-89 were ineffective (Fig. 3A). A 5-min perfusion of fluo-3-labeled rat ZG cells with 5 μM KN93 had no effect on the subsequent [Ca\(^{2+}\)] elevation induced by ANG II (Fig. 3B).

**KN93 inhibits ANG II and ACTH-stimulated aldosterone production in Ca\(^{2+}\)-free medium.** In our experiments, in contrast with publications from Durroux et al. (14), Gallo-Payet et al. (18), and Payet et. al. (41), we could not detect any increase in [Ca\(^{2+}\)], in ZG cells stimulated with ACTH, forskolin, or cBIMPS (Fig. 2). To exclude the possibility that these differences are not connected with the sensitivity of Ca\(^{2+}\) dye, we performed experiments in Ca\(^{2+}\)-free DMEM. ZG cells were preinubicated in Ca\(^{2+}\)-free medium for 6 h. Cells were then stimulated with ANG II, ACTH, or a combination of both with KN93 or KN92 (Fig. 4). Incubation of ZG cells in medium without Ca\(^{2+}\) significantly (4.8 ± 0.5, means ± SD; n = 4) reduced basal aldosterone production. However, ANG II and

**Fig. 2.** Activation of cAMP-dependent protein kinase (PKA) by forskolin or ACTH in rat ZG cells had no effect on [Ca\(^{2+}\)]. Forskolin (10 nM) or ACTH (10 nM), measurement, rat ZG cells were prepared and cultured in coverslips, as described in MATERIALS AND METHODS. After 2 days in culture, cells were washed with PBS and left in serum-free DMEM for 6 h and subsequently loaded with 5 μM fluo-3 AM, and changes in [Ca\(^{2+}\)], were monitored with a confocal laser scanning microscope. Cells were perfused with buffer C containing 5 μM KN93. At indicated times, 10 nM ANG II was added to perfusate. Summary record of 40–50 individual cells from 3 different experiments is shown.

**Fig. 3.** A CaMK inhibitor inhibits ANG II-induced aldosterone production without inhibiting ANG II-induced increases in [Ca\(^{2+}\)]. A: inhibition of ANG II-stimulated aldosterone production from bovine ZG cells by KN93. B: KN93 has no effect on [Ca\(^{2+}\)] elevation in rat ZG cells induced by ANG II. Bovine and rat ZG cells were prepared and cultured in 6-well plates, as described in MATERIALS AND METHODS. After 3 days in culture, cells were washed with PBS and left in DMEM with 0.5% FCS for 6 h, after which medium was replaced by fresh DMEM (control), 10 nM ANG II (ANG II in A), or ANG II in combination with KN93, KN92, or H-89 (all 5 μM). After 1 h of incubation, culture medium was collected for aldosterone RIA. Results are means ± SE of 3 different experiments. *Significant differences (P < 0.05) compared with control values in A. For [Ca\(^{2+}\)], measurement (B), rat ZG cells cultured in coverslips were washed with 5 μM fluo-3 AM, and changes in [Ca\(^{2+}\)], were monitored with a confocal laser-scanning microscope. Cells were perfused with buffer C containing 5 μM KN93. At indicated time, 10 nM ANG II was added to perfusate. Summary record of 40–50 individual cells from 3 different experiments is shown.
ACTH stimulated aldosterone production in a similar way as in the presence of Ca\(^{2+}\), indicating that the ACTH-cAMP pathway was not affected by the absence of extracellular Ca\(^{2+}\). KN93 in these experiments strongly inhibited ANG II- (Fig. 4A) and ACTH-stimulated (Fig. 4B) aldosterone production, indicating that the activation of CaMKs by ACTH is independent of extracellular Ca\(^{2+}\).

Forskolin-, cBIMPS-, and ACTH-stimulated CaMK activity were reduced by preincubation with KN93. In these experiments, as in the in vivo experiments, preincubation of cells with KN93 had no effect on PKA activity (compare P-VASP Western blots in Figs. 1 and 5). To exclude nonspecific phosphorylation of autocamtide-3 by PKA, in a separate experiment autocamtide-3 was phosphorylated with the purified catalytic subunit of PKA. In this experiment, PKA activity assessed by phosphorylation of autocamtide-3 increased only

**Fig. 4.** KN93 inhibits ANG II (A), and ACTH (B) stimulates aldosterone production from bovine ZG cells in Ca\(^{2+}\)-free medium. Bovine ZG cells were prepared and cultured in 6-well plates, as described in MATERIALS AND METHODS. After 3 days in culture, cells were washed with PBS and left in Ca\(^{2+}\)-free DMEM with 0.2% FCS for 6 h. Medium was then replaced by fresh Ca\(^{2+}\)-free DMEM (control), 10 nM ANG II, 10 nM ACTH, or in combination with KN93 or KN92 (both 5 μM). After 1 h of incubation, culture medium was collected for aldosterone RIA. Data are presented as fold increase of aldosterone production, with control taken as 1. Results are means ± SE of 3 different experiments. *Significant differences (P < 0.05) compared with control values.

**Fig. 5.** In vitro CaMK activity in homogenate from bovine ZG cells. Bovine ZG cells were prepared and cultured in 10-cm dishes, as described in MATERIALS AND METHODS. After 3 days in culture, confluent cells were washed with PBS and left in serum-free DMEM for 6 h. Cells were preincubated with H-89 or KN93 (both 5 μM) for 10 min, followed by stimulation with ANG II (10 nM) or forskolin (5 μM; top), and ACTH (10 nM) or Sp-5,6-DCT-cBIMPS (cBIMPS, 100 μM; bottom) for an additional 5 min and finally collected in lysis buffer. CaMK activity was measured using autocamtide-3 as substrate. Activity was calculated by subtracting basal activity (homogenate without autocamtide-3) as background. PKA activity in same samples was evaluated by VASP phosphorylation (P-VASP Western blots in top and bottom). Results are means ± SE of 3 different experiments. *Significant differences (P < 0.05) compared with control values; + significant differences (P < 0.05) compared with forskolin-, ANG II- (top), or ACTH-stimulated (bottom) values.
\( \leq 30\% \) (data not shown) compared with the fivefold increase seen in the forskolin-stimulated homogenate of ZG cells for CaMK activity (Fig. 5), whereas phosphorylation of kemptide (PKA-specific substrate) by the catalytic subunit of PKA increased >20-fold (data not shown), indicating that cAMP-stimulated phosphorylation of autocamtide-3 is CaMK specific.

**Involvement of small GTPases in aldosterone production.** Small GTPases (RhoA, Rac, Cdc42, Rap1, and Rap2) play an important role in many cellular functions, including cytoskeleton reorganization and gene expression. Because both of these parameters are highly important in the regulation of aldosterone production, we tested a possible involvement of GTPases in aldosterone production in bovine ZG cells through the use of pull-down assays (Fig. 6A). The results showed that RhoA activity was strongly stimulated by ANG II, whereas forskolin had no effect, or rather it inhibited RhoA activity, and preincubation with H-89 slightly increased RhoA activity. Cdc42 was activated by ANG II and forskolin, and the forskolin effect was PKA dependent (inhibited by preincubation with H-89). Rac and Rap2 had relatively high constitutive activity, and stimulation with ANG II or forskolin did not significantly
influence their activity. Rap1 was more strongly activated by forskolin than by ANG II and the cAMP analog 8-pCPT-2’-O-Me-cAMP, which specifically binds to Epac and activates Rap1 and did not stimulate PKA (P-VASP Western blot; Fig. 6a). Activation of Rap1 was partly reduced by PKA inhibition (preincubation with H-89).

Direct involvement of Rap1 and RhoA/Rho kinase pathways in aldosterone production from bovine ZG cells was tested in a separate set of experiments (Fig. 6b). 8-pCPT-2’-O-Me-cAMP (200 μM) did not stimulate aldosterone production, and the Rho kinase inhibitor Y-27632 (5 μM) had no effect on ANG II- and forskolin-stimulated aldosterone production (Fig. 6b). In addition, KN93, which inhibits aldosterone production stimulated by ANG II, cBIMPS, ACTH, and forskolin (Figs. 2 and 4a), had no significant effect on the activity of all GTPases tested (data not shown), indicating that they are not directly involved in the regulation of acute aldosterone production.

Activation of cAMP pathway in bovine ZG cells did not stimulate p38 and ERK MAPK. Activation of ERK and p38 MAP kinases plays an important role in the regulation of aldosterone production. ERK and p38 MAPKs have been shown to stimulate CEH activity (8) and regulate [Ca2+]i, by the inhibition of the Na+/Ca2+ exchanger (48). Therefore, we investigated the activation of all three major MAPK pathways (JNK, ERK, and p38) using phosophspecific antibodies in response to ANG II and forskolin stimulation. In all experiments, JNK activity (assessed by the phosophspecific Thr183/Tyr185 antibody) in ZG cells was not changed by ANG II and forskolin stimulation (data not shown). ERK and p38 were activated only by ANG II, but not by forskolin (Fig. 7) or ACTH (data not shown). Two specific ERK inhibitors (U-0126 and PD-98059) totally prevented ERK phosphorylation induced by ANG II stimulation (Fig. 7). However, U-0126 inhibited both ANG II- and forskolin-stimulated aldosterone production, whereas PD-98059 had no significant effect on aldosterone production (Fig. 7). Two inhibitors of p38 MAPK (SB-203580 and SB-202190) reduced the phosphorylation of the p38 substrate MAPKAP2 in response to ANG II without affecting the phosphorylation of p38 MAPK itself (Fig. 7). The p38 MAPK inhibitor SB-202190 also inhibited phosphorylation of HSP-27 and ANG II-stimulated aldosterone production (Fig. 7). Forskolin did not induce p38 MAPK phosphorylation, and p38 MAPK inhibition had no significant effect on forskolin-stimulated aldosterone production (Fig. 7).

Expression of CaMKs in ZG cells and involvement of CaMKII and CaMKK in ANG II and ACTH-stimulated aldosterone production. In earlier studies (16, 42), CaMKII was thought to be responsible for ANG II stimulation of aldosterone production. Later, it was shown that CaMKII activity was responsible for regulation of aldosterone synthase expression in H295R cells (10). Here, we show that rat and bovine ZG cells express all forms of CaMKs (CaMKI, CaMKII, CaMKIV, and CaMKK) at approximately similar levels (Fig. 8). To distinguish between CaMKII and CaMKK effects on aldosterone production stimulated by ANG II or ACTH, we used a recently described, specific CaMKK inhibitor, STO-609 (54), and cell-permeable CNI. A 27-mer CNI is derived from CaMKII inhibitor protein, which specifically inhibits CaMKII activity (7). To generate cell-permeable peptide, an antenapedia sequence (RQIKIWFQNRRMKWKK) was placed NH2-terminal to CNI (antCNI) as described (17). ANG II-stimulated aldosterone production was significantly inhibited by STO-609 and antCNI, whereas ACTH-stimulated aldosterone production was inhibited only by STO-609 (Fig. 9), indicating that ANG II activates both CaMKII and CaMKK, whereas ACTH activates only CaMKK.
Although the regulation of aldosterone production by cAMP and Ca\(^{2+}\) is summarized in recent reviews (19, 37, 46), the downstream signaling of both second messengers is controversial and far from being completely resolved. One of the reasons for controversial data may be connected with the fact that some of the signaling mechanisms generated by cAMP and Ca\(^{2+}\) (activation of MAPKs, PKC, or small GTPases) may be more responsible for other cellular functions, such as proliferation or migration of ZG cells, than directly involved in acute stimulation of aldosterone production. Therefore, in the present work we focused on activation and cross-talk between major serine/threonine protein kinases (PKA, MAPKs, CaMKs) and small GTPases (RhoA, Rac, Cdc42, Rap) in relation to the regulation of acute aldosterone production stimulated by cAMP and ANG II.

ANG II activates ERK and p38 MAPKs in bovine and rat ZG cells and in H295R cells. Activation of ERK is linked not only to cell proliferation (39, 53) but also to phosphorylation and stimulation of CEH activity (8). Activation of p38 is thought to be important in the regulation of [Ca\(^{2+}\)]\(_i\) by inhibition of the Na\(^+\)/Ca\(^{2+}\) exchanger in bovine ZG cells (48) and in the stimulation of aldosterone production by the lipoxygenase pathway in H295R cells (27, 40). However, there is no evidence for cAMP-stimulated activation of MAPK in ZG cells. Therefore, we investigated the activation of ERK and p38 MAPKs in response to ANG II and forskolin stimulation. In our experiments, ERK and p38 were activated only by ANG II, but not by forskolin (Fig. 7). Two ERK inhibitors (U-0126 and PD-98059) completely inhibited ERK phosphorylation, although their effects on aldosterone production were different. U-0126 strongly inhibited ANG II- and forskolin-stimulated aldosterone production, whereas PD-98059 had no significant effect on forskolin-stimulated aldosterone production (Fig. 7). We conclude that p38 MAPK may play a role in ANG II-stimulated aldosterone production. However, MAPKs are not involved in cAMP-stimulated aldosterone production from ZG cells.

Fig. 8. Expression of CaMKs in rat and bovine ZG cells. Rat and bovine ZG cells were cultured in 6-well plates, as described in MATERIALS AND METHODS, washed with PBS, and harvested for Western blot analysis. Protein extracts of ZG cells (30 μg protein) and from whole rat brain (5 μg protein, used as a positive control) were blotted and incubated with corresponding CaMK antibodies. Blots shown are representative of 3 different Western blot experiments. CaM KK, CaMK kinase.

Fig. 9. Inhibition of ANG II- and ACTH-stimulated aldosterone production by specific CaMKII-inhibitory peptide (antCNt) and CaMKK-specific inhibitor (STO-609). Bovine ZG cells were prepared and cultured in 6-well plates, as described in MATERIALS AND METHODS. After 3 days in culture, cells were washed with PBS and left in DMEM with 0.5 FCS for 6 h. Medium was then replaced by fresh DMEM with ANG II, ACTH (both 10 nM), antCNt (2.5 μM), STO-609 (2 μM), or in a combination of inhibitors with ANG II or ACTH. After 1 h of incubation, culture medium was collected for aldosterone RIA. Data are presented as fold inhibition of aldosterone production with ANG II- or forskolin-stimulated aldosterone production taken as 1.0. Results are means ± SE of 3 different experiments. *Significant differences (P < 0.05) compared with control values.

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Rho family GTPases, in addition to actin cytoskeleton reorganization, regulate many other signal transduction pathways such as gene expression, cell cycle progression, microtubule dynamics, cell migration, vesicular transport pathways, and the activity of a variety of enzymes, including cytochrome b558 NADPH oxidase (1, 15, 26, 43, 44). Here, we demonstrated for the first time that the members of Rho family GTPases (RhoA, Rac, Cdc42, Rap1, and Rap2) are highly expressed in ZG cells. From all GTPases tested, only the activation of RhoA, Cdc42, and Rap1 correlated with stimulation of aldosterone production (Fig. 6A). RhoA and Rap1 are probably not directly involved in acute regulation of aldosterone production because a Rho kinase inhibitor and specific activator of Epac/Rap1 (8-pCPT-2′,O-Me-cAMP) had no effect on aldosterone production (Fig. 6B). However, more detailed evaluation of Rho family GTPases in ZG cells is warranted, using dominant negative and constitutively active GTPases.

The main finding of our study is tied to the fact that in ZG cells, CaMK can be activated directly by cAMP and independently of PKA and Rap GTPases. Usually, activation of CaMK requires an increase of [Ca\(^{2+}\)], and there are several reports in the literature that ACTH can increase [Ca\(^{2+}\)], in bovine (34–36), rat, and human ZG cells (14, 18, 35, 41). Nevertheless, experiments studying [Ca\(^{2+}\)], in ZG cells after treatment with ACTH, forskolin, or cAMP analogs have yielded conflicting results. The reason for this is probably connected with different experimental settings, for example, differences in extracellular potassium or Ca\(^{2+}\) concentrations, species differences, or the use of different Ca\(^{2+}\) sensitive dyes or 45Ca\(^{2+}\). For example, Kojima and colleagues (34–36) found an immediate increase in [Ca\(^{2+}\)], in bovine ZG cells after ACTH stimulation, whereas Durroux et al. (14), Gallo-Payet et al. (18), and Payet et al. (41) revealed PKA-dependent increases in [Ca\(^{2+}\)], in rat and human ZG cells only several minutes after ACTH stimulation. In contrast, Iida et al. (32) and Braley et al. (5) did not find any changes in [Ca\(^{2+}\)], in bovine ZG cells after ACTH, forskolin, or cAMP analog stimulation. In our experiments with fluo-3 AM-labeled rat ZG cells, stimulation with ACTH, forskolin, or cBIMPS did not increase [Ca\(^{2+}\)]. However, the same cells showed the expected increase in [Ca\(^{2+}\)], after stimulation with ANG II or 50 mM potassium (Fig. 3). Several other lines of evidence support our conclusion that CaMK can be activated directly by cAMP, independently of PKA and Rap GTPases, and without increasing [Ca\(^{2+}\)]; 1) CaMK inhibitor KN93 strongly reduced forskolin- and ACTH- and cBIMPS-stimulated aldosterone production without affecting PKA activity (Fig. 1); 2) elevation of intracellular cAMP level by forskolin, ACTH, or cBIMPS did not increase [Ca\(^{2+}\)] (Fig. 2); 3) ACTH stimulated aldosterone production from bovine ZG cells in the absence of external Ca\(^{2+}\) (Fig. 4); 4) in in vitro kinase assays, forskolin, ACTH, or cBIMPS stimulated CaMK activity that was inhibited by KN93, but not by H-89 (Fig. 5); and 5) cAMP activated Rap1, but the activation of Rap1 via Epac did not stimulate aldosterone production (Fig. 6B).

PKA and CaMKs may act synergistically, and in different cell types both kinases are known to phosphorylate a cAMP-responsive element-binding protein at Ser\(^{133}\) (24, 31, 33). In neuronal cells, PKA activates the IP\(_3\) receptor by phosphorylation and increases [Ca\(^{2+}\)], (52, 53, 55), which in turn may stimulate CaMKs. However, in ZG cells, cAMP did not increase [Ca\(^{2+}\)], in the presence (Fig. 2B) or absence (data not shown) of extracellular Ca\(^{2+}\), and ACTH stimulates aldosterone production in Ca\(^{2+}\)-free medium (Fig. 4B). An opposite effect of PKA was shown in Jurkat cells and primary cultures of hippocampal neurons, where it may inhibit CaMKI and -IV activity by direct phosphorylation (Ser\(^{458}\) and Thr\(^{108}\)) and inhibition of CaMKK activity (56). cAMP-mediated but PKA- and Rap1-independent effects have been proposed in several cell types (12, 13). In ovarian granulosa cells, activation of PKB and glucocorticoid-induced kinase stimulated by cAMP was not mediated by PKA (23, 25). Inhibition of IL-5 secretion from human T lymphocytes and activation of the Ras-ERK pathway in melanocytes and melanoma cells by cAMP were also independent of PKA activity (7, 47, 48). Here, we present new data indicating that CaMK can also be activated by cAMP independently of PKA and Rap1 activity. Sequence analysis of all CaMKs, including the recently cloned CaMKI-γ (51) and different splice variants of CaMKII-γ (22), did not show any homology with known cyclic nucleotide-binding domains; therefore, direct interaction of cAMP with CaMKs appears to be unlikely.

At least three different classes of proteins have cAMP-binding properties. They include catabolite gene activator protein (CAP)-related proteins (regulatory subunits of PKA, cyclic nucleotide-gated channels, and Epacs), proteins with GAF domains (cGMP-specific and stimulated phosphodiesterases, Anabaena adenylate cyclases, and Escherichia coli FhlA), and the plasma membrane cAMP receptors of Dictyostelium (13). Recently, several new cAMP-binding proteins were identified by a Basic Local Alignment Search Tool search in databases by using cyclic nucleotide-binding domains of CAP proteins as the query sequences (13). A new Rap-specific GEF protein, neuropathy target esterase, and a protein from a human brain cDNA library (KIAA0313) were found by this method (13). Other methods developed for finding new cAMP-binding proteins include pull-down assays using cAMP-linked agarose beads or photolabeling with cAMP analogs. All of these methods have certain advantages and limitations and still leave open the question of the existence of unknown cAMP-binding proteins. Here, we presented strong evidence that ZG cells, in addition to PKA and Epac/Rap proteins, may have an additional cAMP mediator and/or target that modulates CaMKK activity.

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**REFERENCES**


