cGMP-dependent Protein Kinase Type II Regulates Basal Level of Aldosterone Production by Zona Glomerulosa Cells without Increasing Expression of the Steroidogenic Acute Regulatory Protein Gene*

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The renin-angiotensin-aldosterone system plays a pivotal role in the regulation of salt and water homeostasis. Here, we demonstrate the expression and functional role of cGMP-dependent protein kinases (PKGs) in rat adrenal cortex. Expression of PKG II is restricted to adrenal zona glomerulosa (ZG) cells, whereas PKG I is localized to the adrenal capsule and blood vessels. Activation of the aldosterone system by a low sodium diet up-regulated the expression of PKG II, however, it did not change PKG I expression in adrenal cortex. Both, activation of PKG II in isolated ZG cell and adenoviral gene transfer of wild type PKG II into ZG cells enhanced aldosterone production. In contrast, inhibition of PKG II as well as infection with a PKG II catalytically inactive mutant had an inhibitory effect on aldosterone production. Steroidogenic acute regulatory (StAR) protein that regulates the rate-limiting step in steroidogenesis is a new substrate for PKG II and can be phosphorylated by PKG II *in vitro* at serine 55/56 and serine 99. Stimulation of aldosterone production by PKG II in contrast to stimulation by PKA did not activate StAR gene expression in ZG cells. The results presented indicate that PKG II activity in ZG cells is important for maintaining basal aldosterone production.

The renin-angiotensin-aldosterone system plays a pivotal role in the regulation of salt and water homeostasis by promoting the constriction of arterioles within the renal and systemic circulation and reabsorption of sodium in proximal segments of the nephron through angiotensin II (Ang II).[1] Ang II also stimulates aldosterone production from adrenal zona glomerulosa (ZG), which promotes the reabsorption of sodium, by activating sodium-potassium ATPases and epithelial sodium channels (reviewed in Ref.1).

Regulation of aldosterone production is under the multifunctional control of different factors among which the most important are adrenocorticotropin (ACTH), Ang II, and K^+. These factors utilize a variety of transduction mechanisms, including activation of cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and regulation of cytosolic free calcium.

Acute stimulation of steroid production requires both the synthesis of new proteins and protein phosphorylation. The delivery of the steroid substrate cholesterol from the outer to the inner mitochondrial membrane, a process mediated by the steroidogenic acute regulatory protein (StAR), represents the rate-limiting step for the production of aldosterone and other steroids (reviewed in Ref. 2). StAR is a phosphoprotein that has several putative phosphorylation sites for different protein kinases (cAMP- and cGMP-dependent protein kinases, PKC). However, until now only PKA-specific phosphorylation at serine 56 and serine 194 of StAR has been demonstrated and shown to increase the delivery of sterol substrate and, in part, to account for the immediate effects of cAMP on steroid production (3).

Although multiple factors control aldosterone production, only natriuretic peptides (NPs) and nitric oxide (NO), which have effects partially mediated by the second messenger cGMP, are potent inhibitors of agonist-induced aldosterone synthesis in ZG cells (4–6).

Intracellular cGMP effects can be mediated by a number of effectors, including cGMP-gated channels, cGMP-dependent protein kinases, and cGMP-regulated phosphodiesterases (reviewed in Refs. 7 and 8). Several reports indicate that cGMP-driven inhibition of ACTH-stimulated aldosterone synthesis is, at least in part, mediated by cGMP-stimulated phosphodiesterase (PDE 2) that is expressed at high levels in ZG cells (9, 10).

Mammalian PKG exists as two major forms, PKG I, a soluble enzyme consisting of α and β isoforms derived from alternative splicing from one gene, and PKG II, a myristoylated, membrane-associated form derived from a second gene (7, 11). One of the major functions of PKG I is the regulation of intracellular Ca^{2+}, which inhibits vascular smooth muscle contraction, platelet aggregation, and endothelial cell permeability (7, 12).

¹ The abbreviations used are: Ang II, angiotensin II; ACTH, adrenocorticotropic hormone; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PDE, phosphodiesterase; sGC, soluble guanylyl cyclase; StAR, steroidogenic acute regulatory protein; VASP, vasodilator-stimulated phosphoprotein; ZG, zona glomerulosa; NP, natriuretic peptide; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; 8-pCPT-cGMP, 8-*para*-chlorophenyl-thio-cGMP; GST, glutathione *S*-transferase; MS, mass spectrometry; RIA, radioimmunoassay; WT, wild type.
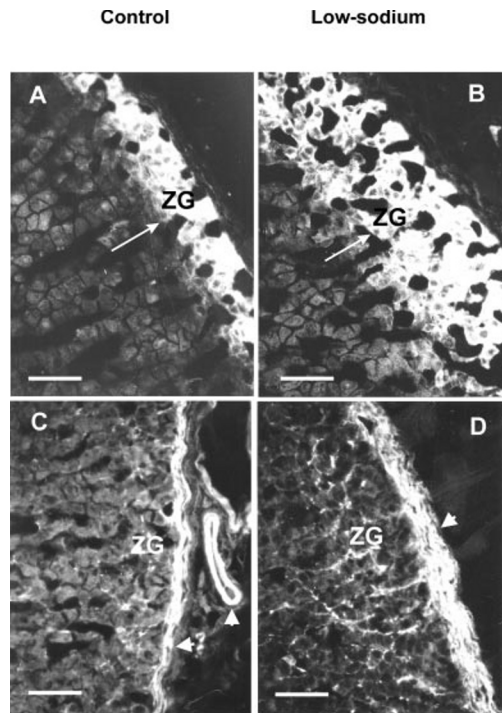
Control   Low-sodium

FIG. 1. **Localization of PKG II and PKG I in adrenal cortex of control and low sodium diet fed rats.** PKG II immunoreactivity in the adrenal cortex was observed only in zona glomerulosa (*ZG*) cells (*arrow* in *A*), whereas that of PKG I was primarily restricted to the adrenal capsule and blood vessels (*arrowheads* in *C*). A low sodium diet that leads to hyperplasia and hypertrophy of ZG (24) increased staining of PKG II (*arrow* in *B*) but not PKG I (*D*) in adrenal ZG cells. Shown are representative results from four different rats. *Bars* in *A–D*, 50 μm.

In contrast to PKG I, PKG II often has a quite different localization and certain distinct and specific functions. Included in the few known PKG II functions are the phosphorylation and activation of the cystic fibrosis transmembrane conductance regulator in intestinal mucosa (13), inhibition of renin release from kidney juxtaglomerular cells (14, 15), stimulation of Ca^{2+} reabsorption in the distal nephron (16), and promotion of bone endochondral ossification at the ground plates (17).

Here, we demonstrate the expression and functional implication of PKG II in rat adrenal cortex. Expression of PKG II in adrenal cortex is restricted to ZG cells, whereas PKG I is localized in the adrenal capsule and blood vessels. We present strong evidence that PKG II serves as an important regulator of basal aldosterone production and that the stimulation of aldosterone production by PKG II correlates with the phosphorylation of StAR protein but not with an activation of StAR gene expression in ZG cells.

EXPERIMENTAL PROCEDURES

Animal Experiments and Immunohistochemistry—Male Sprague-Dawley rats (200–300 g) were used in all experiments. Low sodium diet rats were kept for 5 days on a diet containing 0.02% NaCl (0.6% NaCl, is a standard diet). After 5 days rats were killed by decapitation, and adrenal glands were dissected to the adrenal capsule with zona glomerulosa (cortex) and remaining adrenal tissues (medulla) and used for Western blotting. For immunohistochemistry, rats were perfused through the abdominal aorta with 4% paraformaldehyde in PBS, and sections of adrenal gland were prepared from three control and three low sodium diet rats. The sections were incubated with affinity-purified PKG II (1:100) or PKG I (1:200) (18) antibodies followed by Cy3-labeled (1:500) secondary goat anti-rabbit antibody (Dianova, Hamburg, Germany). The specificity of immunofluorescent staining was verified by pre-absorption of affinity-purified antibodies with the respective recombinant PKG II or PKG I proteins (data not shown) as described previously (19). Animal experiments were performed in accordance with the German animal protection law.
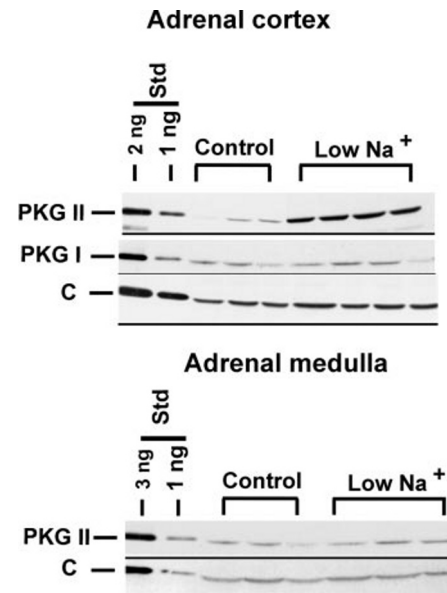
Adrenal cortex

Adrenal medulla

FIG. 2. **A low sodium diet activated the expression of PKG II in rat adrenal cortex but not adrenal medulla.** Adrenal capsule with ZG (*adrenal cortex*) and remaining tissues, including zona fasciculata and reticularis, and adrenal medulla (*adrenal medulla*) were separated from control and low sodium diet-fed rats as described under "Experimental Procedures" and processed for Western blot analysis. A 5-day low sodium diet strongly activated expression of PKG II but not PKG I and the C-subunit of PKA only in ZG. The blots shown are representative of three different experiments.

Western Blot Analysis—Adrenal cortex and medulla were homogenized in PBS containing protease inhibitors (Complete™, Roche Applied Science, Mannheim, Germany). SDS gel loading buffer was added to the homogenate and directly to freshly isolated and cultured ZG or NCI cells. Samples were analyzed by Western blot using the following antibodies: affinity-purified against PKG II, 1:1000; PKG I, 1:1000; antiserum against C-subunit of PKA, 1:3000 (20); antiserum against StAR, 1:500 (generous gift from Dr. D. Stocco). PKG and PKA activities were evaluated by the phosphorylation of the well known PKA and PKG substrate vasodilator-stimulated phosphoprotein (VASP) detected by monoclonal anti P-Ser^{239}-VASP antibody (1 μg/ml) (21, 22). For visualization of the signal, goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase was used as secondary antibodies, followed by ECL detection (Amersham Biosciences, Freiburg, Germany).

Preparation of Zona Glomerulosa Cells—Cells were prepared from a dissected rat adrenal capsule with ZG by collagenase digestion and mechanical disaggregation as described previously (23). For experiments in freshly isolated cells, the cells were suspended in DMEM (50–100 × 10^5 cells/sample) and preincubated for 1 h at 37 °C in the cell culture incubator. After this resting period, cells were resuspended in 1 ml of fresh DMEM with different combinations of substances to be tested: ACTH 1–24 peptide (Organon, Toronto, Canada), ODQ (Calbiochem, Alexis, Laeufelfingen, Switzerland), forskolin (Sigma, Deisenhofen, Germany), 8-pCPT-cGMP, R_p-8-pCPT-cGMPS (BioLog, Bremen, Germany). For primary culture, cells were plated into 6-well plates at a density of 7–10 × 10^4 cells/well. After 2 days cells were directly used for experiments of aldosterone production or were first infected with adenovirus containing cDNA of rat PKG II or adenovirus encoding a catalytically inactive form of rat PKG II (PKG II K482A).

Experiments on NCI-h295R Cells—NCI adrenocortical cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM/Ham's F-12 medium (Sigma) supplemented with 1% insulin-transferrin-sodium selenite premixed (ITS, Roche Applied Science) in 6-well plates. The cells were grown until confluence, washed with PBS, and fresh media with tested substances was added to cells for 6 h, then media was removed and used for aldosterone determination.

Adenoviral Infection of Zona Glomerulosa Cells and Construction of PKG II K482A Mutant—An adenoviral vector containing the cDNA of rat PKG II (13) was used to overexpress PKG II in cultured rat ZG cells. As a control, a vector encoding a catalytically inactive rat PKG II (PKG II-K482A) was used. The inactive mutant was generated using PCR-mediated site-directed mutagenesis to replace the essential Lys^{482} with Ala within the ATP binding site of PKG II, confirmed by sequencing,

Regulation of Aldosterone Production by PKG

FIG. 3. **Effect of PKG II activity on aldosterone production from freshly isolated rat ZG cells.** *A*, dose-dependent effect of activation (8-pCPT-cGMP) and inhibition (Rp-8-pCPT-cGMPS) of PKG II on basal level of aldosterone production. *B*, inhibition of basal aldosterone production by ODQ. *C*, additive effect of PKG II activity on stimulated aldosterone production. Cells were prepared as described under "Experimental Procedures" and divided into tubes containing about 200,000 cells per tube. After a 1-h resting period in DMEM, the cells were centrifuged and resuspended in 1 ml of DMEM with indicated concentrations of 8-pCPT-cGMP or Rp-8-pCPT-cGMPS (*A*) and of ODQ (*B*), or the cells were incubated with 500 μM 8-pCPT-cGMP or Rp-8-pCPT-cGMPS for 20 min, then aldosterone production was stimulated by 10 nM ACTH, 10 μM forskolin, or 10 nM Ang II (taken as 100% in *C*). After 1 h of incubation the cells were centrifuged and aldosterone concentration in supernatant was determined by RIA. Results are mean ± S.E. of three different experiments. *, significant differences from control values in *A* and *B*, and from stimulated values of aldosterone production in *C* ($p < 0.05$).
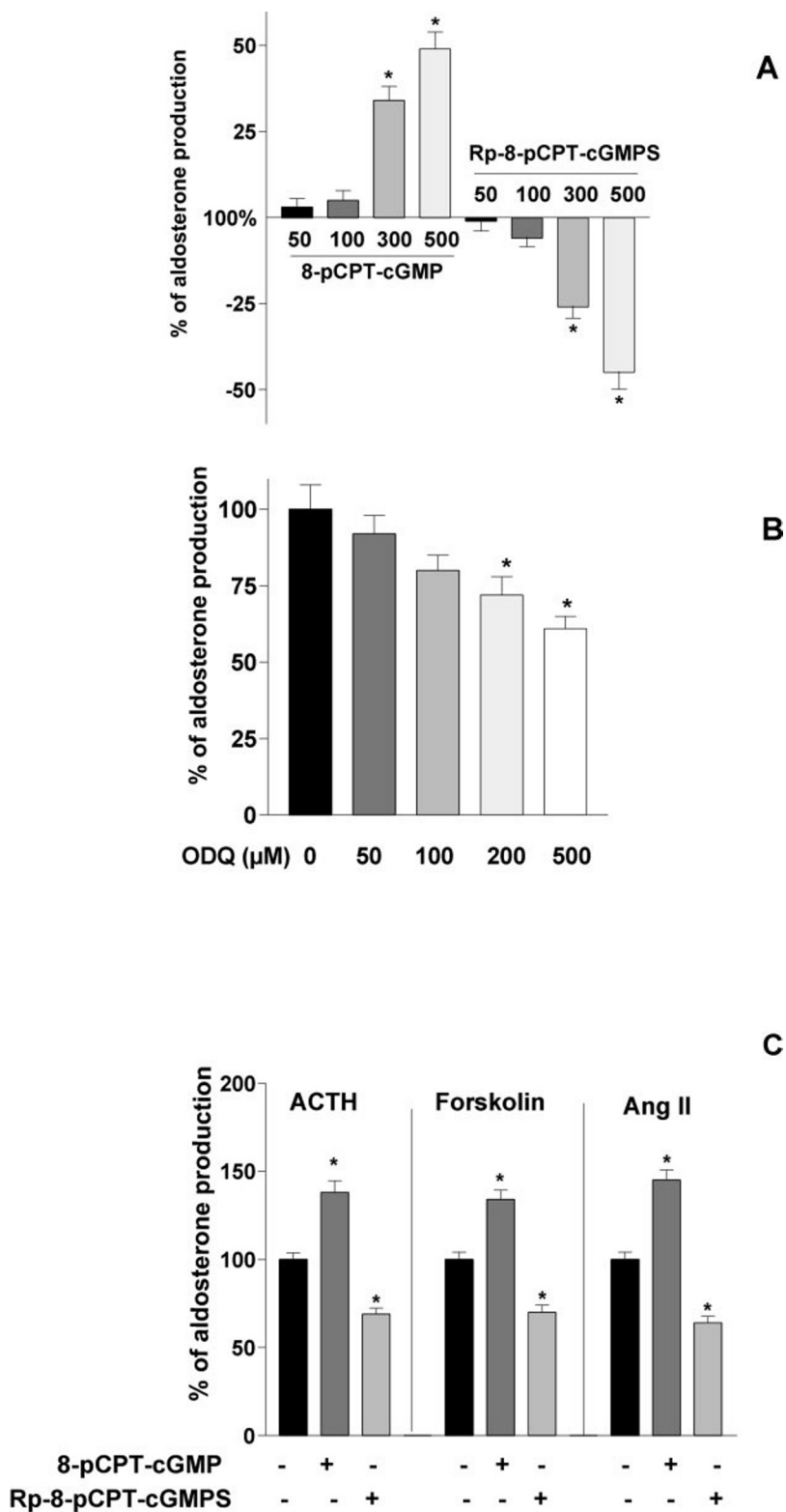
and inserted into an adenoviral vector using the method described previously (13). ZG cells cultured for 2 days were infected with adenoviral vectors (10^{11} particles/ml) then incubated for a further 24 h for PKG II expression. Adenovirus were then removed, and 1 ml of DMEM with or without test substances was added to cells for 1 h, then medium was removed and used for aldosterone determination, and cells were harvested for Western blot analysis of PKG II expression and VASP phosphorylation.

In Vitro Phosphorylation of StAR Protein—For overexpression and protein purification the full-length StAR protein cDNA was cloned by reverse transcription-PCR using published sequence information (24)

and subcloned into the pEBG vector (generous gift from Dr. A. Avots (25)) as a glutathione *S*-transferase (GST) fusion protein. All constructs were confirmed by sequence analysis. Human embryonic kidney 293 cells were transfected with pEBG-StAR, and GST-StAR protein was purified in a glutathione column and phosphorylated *in vitro* by PKG II and the catalytic subunit of PKA type II as described (26). Proteins were separated by SDS-PAGE, and ^{32}P incorporation was visualized by autoradiography.

Mass Spectrometry Analysis—Full-length rat StAR cDNA was cloned into the *Bam*HI/*Eco*RI sites of pGEX4T1 vector (Amersham Biosciences) to generate GST-StAR fusion protein. Expression and purifi-
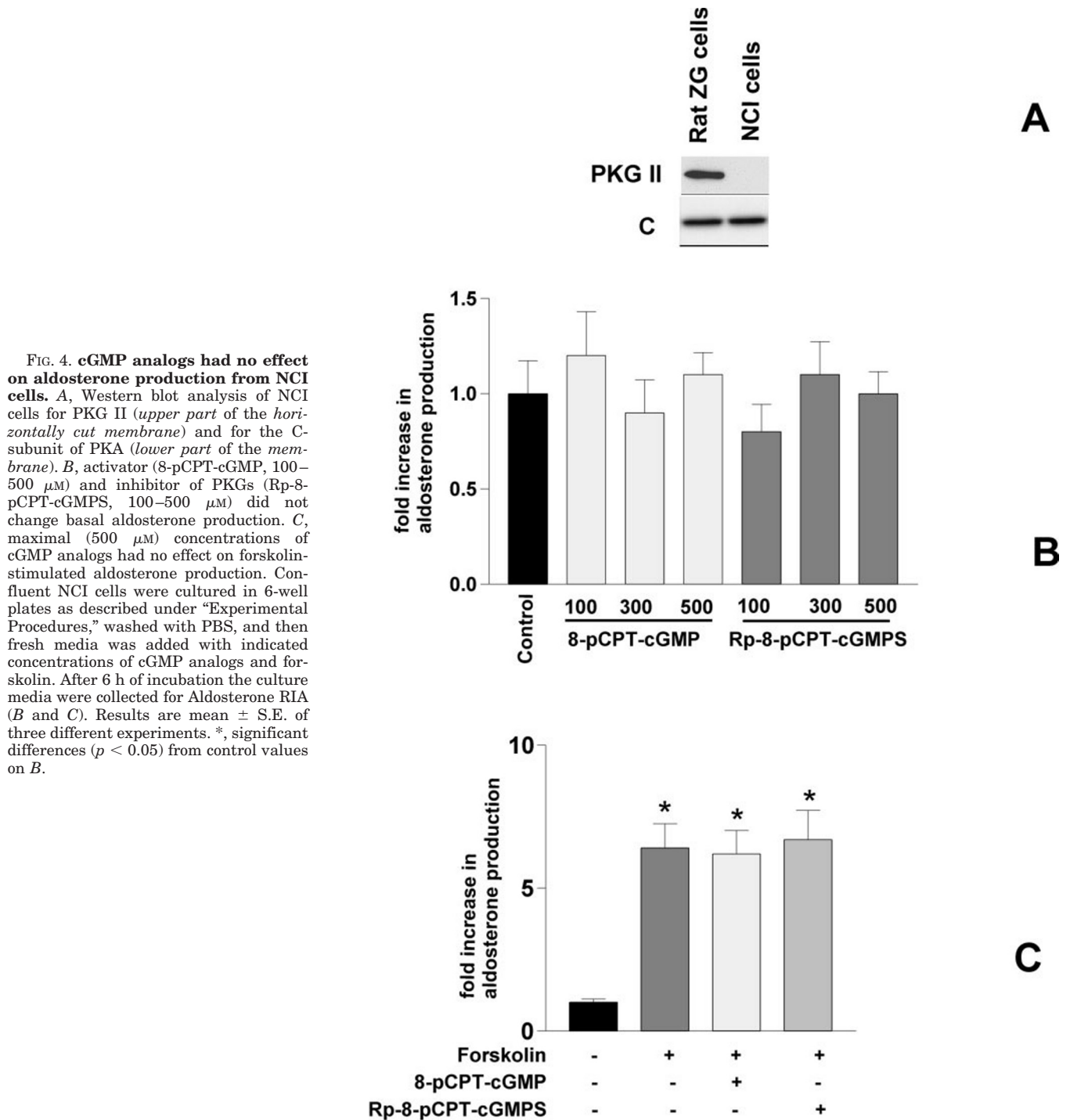
FIG. 4. **cGMP analogs had no effect on aldosterone production from NCI cells.** *A*, Western blot analysis of NCI cells for PKG II (*upper part of the horizontally cut membrane*) and for the C-subunit of PKA (*lower part of the membrane*). *B*, activator (8-pCPT-cGMP, 100–500 μM) and inhibitor of PKGs (Rp-8-pCPT-cGMPS, 100–500 μM) did not change basal aldosterone production. *C*, maximal (500 μM) concentrations of cGMP analogs had no effect on forskolin-stimulated aldosterone production. Confluent NCI cells were cultured in 6-well plates as described under "Experimental Procedures," washed with PBS, and then fresh media was added with indicated concentrations of cGMP analogs and forskolin. After 6 h of incubation the culture media were collected for Aldosterone RIA (*B* and *C*). Results are mean ± S.E. of three different experiments. *, significant differences ($p < 0.05$) from control values on *B*.

cation of the GST fusion protein was performed according to the manufacturer's protocol. Purity was analyzed by examination of Coomassie Blue-stained SDS-PAGE. Purified StAR protein was phosphorylated *in vitro* by the catalytic subunit of PKA type II, and separated by SDS-PAGE. Gel pieces were washed sequentially for 10 min in tryptic digestion buffer (10 mM NH_4HCO_3) and digestion buffer:acetonitrile (1:1). These steps were repeated three times and led to a shrinking of the gel. It was reswollen with 2 μl of protease solution (trypsin at 0.05 μg/μl) in digestion buffer and incubated overnight at 37 °C. Analysis of the resulting peptides was carried out using a nano-HPLC system coupled directly to an electrospray ionization-ion trap mass spectrometer equipped with a custom-built nano-electrospray ion source (LCQ™ Classic, Thermo Finnigan, San Jose, CA). 15 μl of 5% (v/v) formic acid was added to the gel pieces, and the peptides were extracted by sonication for 15 min. The extraction step was repeated once. The supernatants were transferred to glass tubes, and the peptides were automatically flushed and preconcentrated on a μC_{18} pre-

column (Nano-Precolumn™, 0.3-mm inner diameter × 1 mm, C18 PepMap™, LC Packings Dionex, Idstein, Germany) for 10 min with 0.1% (v/v) trifluoroacetic acid and a flow of 40 μl/min. Tryptic peptides were injected automatically on the reversed-phase C_{18} column (75-μm inner diameter × 250 mm, C18 PepMap™, 5-μm particle size, LC Packings Dionex) using the Switchos™ system (LC Packings Dionex). Separation of the peptides was carried out in a gradient consisting of 0.5% (v/v) formic acid (solvent A) and 0.5% formic acid 84% acetonitrile (solvent B) with 5–15% B in 10 min, 15–20% B in 10 min, 20–50% B in 70 min, and 50–100% B in 5 min. The flow rate was adjusted from 200 μl/min to 160 nl/min using a precolumn split. Eluting peptides were transferred online to a heated capillary (Pico Tip™, FS360-20-10, New Objective Inc., Cambridge, MA) of an ion trap mass spectrometer (LCQ™ Classic, Thermo Finnigan). The following electrospray ionization parameters were used: spray voltage, 1.8–2.15 kV; capillary temperature, 200 °C; capillary voltage, 42 V; tube lens offset voltage, 30 V; and the electron multiplier at −950 V. The collision energy was set

FIG. 5. **Adenoviral gene transfer of PKG II enhanced cGMP-dependent stimulation of aldosterone production from 2-day cultured rat ZG cells.** Cells cultured in 6-well plates for 2 days as described under "Experimental Procedures" were subsequently infected with adenovirus containing wild type PKG II (*PKG II-WT*) or the catalytically inactive PKG II-K482A mutant. After 24-h infection, the media was replaced by fresh DMEM, or DMEM containing the shown concentrations of forskolin or 8-pCPT-cGMP for 1 h, after which time the culture media was collected for aldosterone RIA (*upper panel*), and cells were harvested for Western blot analysis (*lower panel*). Western blots demonstrated the level of endogenous and expressed PKG II, and the phosphorylation of VASP as a reporter of the activity of PKA (stimulated by forskolin) or PKG II (stimulated by 8-pCPT-cGMP). Aldosterone production from forskolin-stimulated cells was not affected by the infection procedure. Similarly, Western blots also showed that these adenoviral infections did not affect forskolin stimulation of VASP phosphorylation by PKA. 8-pCPT-cGMP dose-dependently activated aldosterone production in non-infected cells, and this effect was strongly enhanced by expression of exogenous PKG II-WT, whereas it was inhibited by PKG II-K482A. Results are mean ± S.E. of three different experiments. *, significantly different ($p < 0.05$) aldosterone production, in comparison to the respective non-infected cells shown in each set of three conditions. §, significantly different ($p < 0.05$) with respect to control (no 8-pCPT-cGMP) non-infected cells.
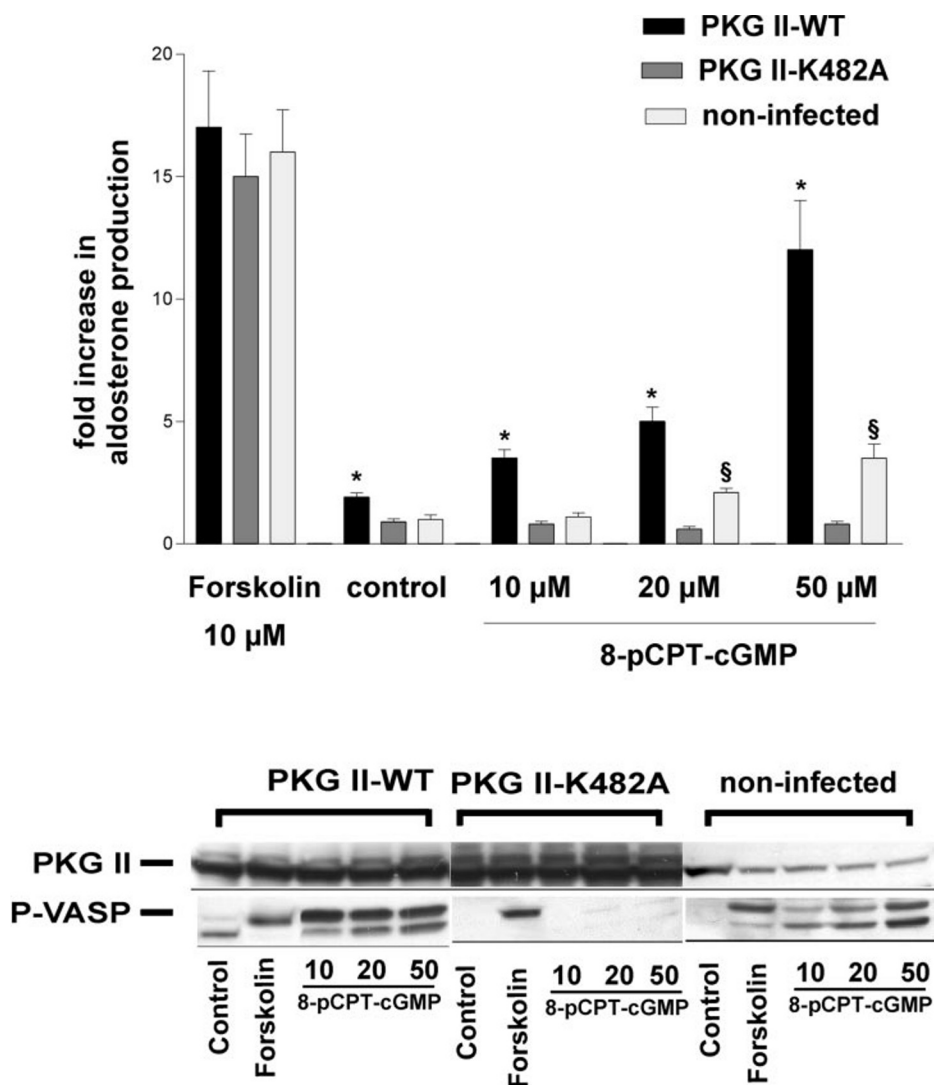
automatically depending on the mass of the parent ion. Gain control was set to 10^7. The data were collected in the centroid mode using one MS experiment (Full-MS) followed by three MS/MS experiments of the three most intensive ions (intensity at least 3×10^5). "Dynamic exclusion" was used for data acquisition with an exclusion duration of 5 min and an exclusion mass width of $\pm$ 1.5 Da.

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 triplicate experiments. Values are expressed as the mean ± S.E. Differences between groups were analyzed by Student's *t* test. Analysis of variance was used for the dose-response experiments. $p < 0.05$ was considered statistically significant.

RESULTS

Localization of PKGs in Adrenal Gland and Experiments on Low Sodium Diet—In adrenal cortex PKG II is localized in ZG cells (Fig. 1, *A* and *B*), whereas localization of PKG I is restricted to the adrenal capsule and blood vessels (Fig. 1, *C* and *D*). Low sodium intake resulted in the activation of the renin-angiotensin system, increase of plasma aldosterone levels, hyperplasia and hypertrophy of ZG cells, and an increase of cytochrome P450 aldosterone synthase activity, and StAR protein expression (27–29). In our experiments a low sodium diet activated the expression of PKG II, whereas the expression levels of PKG I and the C-subunit of PKA did not change during the experiment. The increase in PKG II expression was restricted to the ZG and was not observed in adrenal medulla

(Fig. 2). Low sodium diet also increased PKG II-positive cells in ZG without changing PKG I expression and localization (Figs. 1 and 2).

Regulation of Basal Aldosterone Production by PKG II—cGMP generated upon activation of soluble or particulate guanylyl cyclases is a well known inhibitor of aldosterone production by stimulation of PDE II (9, 10). Therefore, in our experiments we used selective PKG activator (8-pCPT-cGMP) and inhibitor (Rp-8-pCPT-cGMPS) in concentrations that do not affect phosphodiesterases (30). The absolute values of aldosterone production were consistent with published results; however, the values are expressed as percent or -fold increase from control to compare data from different cell preparations. In freshly isolated ZG cells activation of PKG II stimulated and inhibition of PKG II inhibited basal aldosterone production dose-dependently (Fig. 3*A*). The inhibitory effect of Rp-8-pCPT-cGMPS on aldosterone production was mimicked by ODQ, the inhibitor of soluble guanylyl cyclase (Fig. 3*B*).

To investigate whether PKG II effects on aldosterone production are independent of other stimuli the following experiments were performed on freshly isolated ZG cells in which the aldosterone production was stimulated by ACTH, forskolin, or Ang II. Stimulation of ZG cells with ACTH or forskolin resulted in ~6-fold, and with Ang II ~3-fold, increase of aldosterone production. Further activation of PKG II with 8-pCPT-cGMP, like in the experiments on basal aldosterone production (com-

pare with Fig. 3*A*), is enhancing stimulated aldosterone production (138 ± 7% for ACTH, 134 ± 6% for forskolin, and 145 ± 8% for Ang II, $p < 0.05$). Inhibition of PKG II with Rp-8-pCPT-cGMPS is inhibiting stimulated aldosterone production (69 ± 7% for ACTH, 70 ± 6% for forskolin, and 64 ± 5% for Ang II, $p < 0.05$) (Fig. 3C), indicating that PKG II effects in ZG cells are independent from other stimuli.

cGMP Analogs Had No Effect on Aldosterone Production from NCI Cells—To demonstrate that specific effects of cGMP analogs on aldosterone production are mediated by PKG II, NCI cells, a cell line that secretes aldosterone in a regulated manner and does not express PKG II (Fig 4*A*), were used in the same way as rat ZG cells experiments. 8-pCPT-cGMP and Rp-8-pCPT-cGMPS in the same concentrations (100, 300, and 500 μM) had no effect on aldosterone production (Fig 4*B*). Forskolin stimulated aldosterone production 6.5 ± 1.8-fold (values comparable with rat ZG cells), however, in contrast to rat ZG cells (Fig. 3*C*) 8-pCPT-cGMP and Rp-8-pCPT-cGMPS (500 μM) had no additional effects on forskolin-stimulated aldosterone production (Fig. 4*C*).

Gene Transfer of PKG II Enhanced cGMP-dependent Aldosterone Production from ZG Cells—To obtain direct evidence that PKG II stimulates aldosterone production, ZG cells were infected with adenoviral vectors expressing either wild type PKG II (PKG II-WT) or, as control, a catalytically inactive PKG II-K482A mutant. Overexpression of PKG II-WT greatly increased the ability of 8-pCPT-cGMP to stimulate aldosterone production. In contrast, the aldosterone production caused by 8-pCPT-cGMP stimulation of endogenous PKG II was suppressed by overexpression of the catalytically inactive PKG II-K482A mutant. Overexpression of PKG II-WT and PKG II-K482A had no effect on forskolin-stimulated aldosterone production (Fig. 5, *upper panel*) and, thus, apparently did not affect PKA-mediated effects of cAMP elevated by forskolin. Western blot analyses confirmed these conclusions. Western blots (Fig. 5, PKG II Western blot) demonstrated the amount of endogenous PKG II in ZG cells and the higher amounts of overexpressed PKG II-WT and mutant PKG II-K482A. The activity of PKA and PKG II was examined by VASP phosphorylation using the phosphorylation-specific antibody, which recognized VASP only when it is phosphorylated at Ser[239] (Fig. 5, P-VASP Western blot). Phosphorylation of VASP at Ser[157] altered the apparent molecular mass of VASP in SDS-PAGE from 46 to 50 kDa (*double band* in Fig. 4, P-VASP Western blot). Overexpression of PKG II-K482A had no effect on PKA activity (*forskolin bands* on P-VASP Western blot, Fig. 5), and inhibitory effect of PKG II-K482A correlates with inhibition of PKG II activity (Fig. 5, P-VASP Western blot).

In Vitro Phosphorylation of StAR Protein by PKG II and Identification of Phosphorylation Sites—Activation of steroidogenesis and aldosterone production by ACTH is directly correlated with phosphorylation of StAR protein by PKA (3). To investigate whether PKG II-stimulated aldosterone production from ZG cells also correlated with StAR protein phosphorylation and whether StAR is a substrate for PKG II, we cloned the full-length StAR cDNA from rat adrenal gland and overexpressed it as a GST fusion protein in HEK-293 cells. After purification recombinant StAR protein was phosphorylated by the catalytic subunit of PKA and by PKG II. After exposure of the membrane, StAR protein was identified by Western blot analysis (Fig. 6). Incorporation of phosphate with approximately similar levels was observed after 30 min of incubation with both kinases.

For identification of PKG II and PKA phosphorylation sites, rat StAR cDNA was cloned in PGEX4T1 vector, and, after protein purification, it was phosphorylated *in vitro* by PKA and
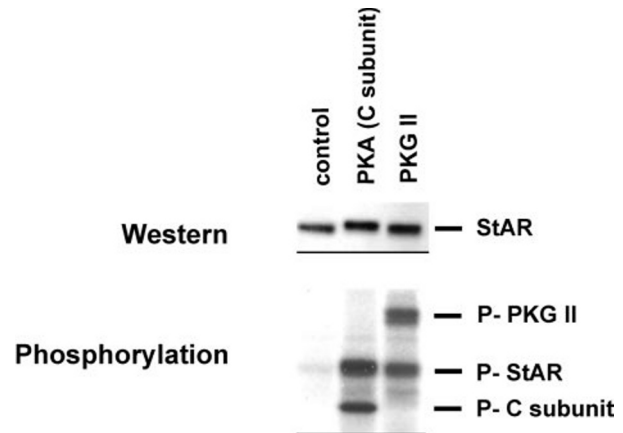

FIG. 6. *In vitro* **phosphorylation of rat StAR protein by PKG II and PKA.** Purified recombinant StAR protein was phosphorylated by PKG II or the C-subunit of PKA (each 0.05 μM) in a total volume of 20 μl for 30 min as described under "Experimental Procedures." The autoradiogram (*lower panel*) reveals phosphorylation of StAR by both kinases. The bands for autophosphorylated PKG II (*P-PKG II*) and PKA (*P-C subunit* of PKA) are indicated. The corresponding anti-StAR immunoblot (*upper panel*) demonstrates immunoreactive protein corresponding to the phosphoprotein. The blots are representative of two separate experiments.

PKG II. Rat StAR contains several PKA and PKG consensus motifs for serine phosphorylation: serine 55/56 (RRRSS), serine 99 (KKES), serine 195 (KRRGS), and serine 276 (KRKLES). In the present study, phosphorylation of amino acids was determined by mass spectrometry. A complete trypsin digest of StAR, phosphorylated by either PKA or PKG, was fractionated on a nano-HPLC connected online to an ion-trap mass spectrometer. The resulting MS/MS spectra of the phosphopeptides of rat StAR phosphorylated by PKA led to the unequivocal identification of serine 55/56 and serine 194 in rat StAR, corresponding to the human serine 56/57 and serine 195 phosphorylation sites described earlier (3). In addition, another yet unknown phosphorylation site at serine 276 was detected. This site is not conserved among all species and absent, for example, in mouse. Mass spectrometry analysis of rat StAR phosphorylated by PKG detected the already known phosphorylation site for PKA at serine 55/56 and a new, yet predicted site at serine 99 (Fig. 7).

Activation of PKG II in ZG Cells Increased Aldosterone Production but Not StAR Protein Expression—The increase of aldosterone production in ZG cells after stimulation by cAMP directly correlated with activation of StAR gene expression (2, 24). In ZG cells activation of PKG II, although to a lesser degree than activation of PKA, also stimulated aldosterone production, which correlated with the phosphorylation of StAR protein (Fig. 8). We then investigated whether long term (24 h) activation of PKG II, like activation of PKA, would stimulate StAR gene expression. After 2 days in culture, cells were stimulated by 8-pCPT-cGMP or ACTH for an additional 24 h, then cell supernatants were collected for aldosterone determination (Fig. 8*A*), and the expression of PKG II and StAR protein were examined by Western blot. Phosphorylation of VASP was used as a marker of PKA and PKG II activity (Fig. 8*B*). ACTH, by activation of PKA, strongly increased both aldosterone production and StAR protein expression. 8-pCPT-cGMP activation of PKG II increased aldosterone production ~5-fold at 24 h (Fig. 8 *A*) but had no effect on StAR protein expression (Fig. 8*B*).

DISCUSSION

Our results demonstrate that PKG II has a stimulatory effect on aldosterone production from adrenal ZG cells, in addition to
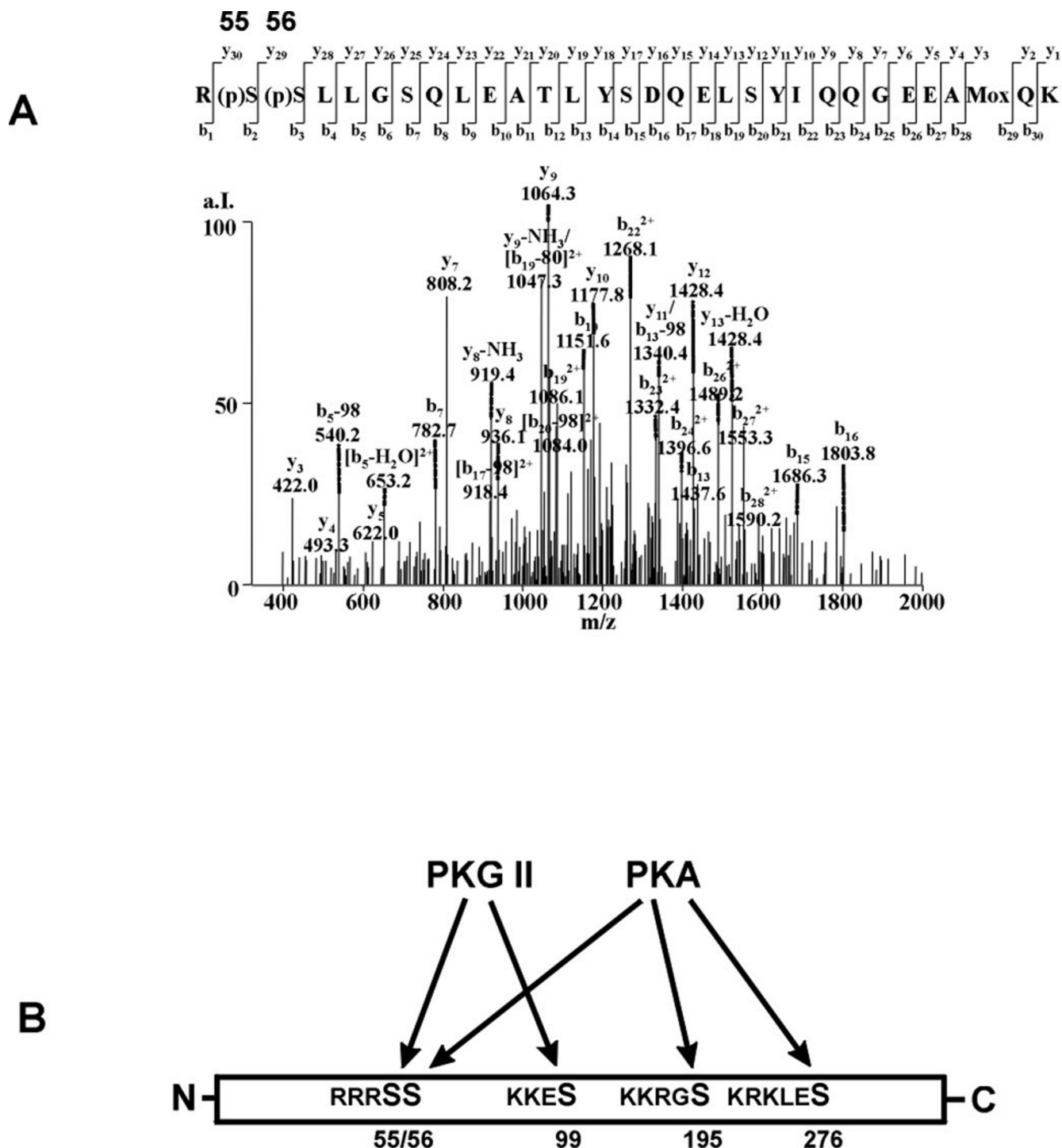
FIG. 7. **Phosphorylation sites in StAR.** *A*, fragment ion spectrum of the phosphorylated peptide RSSLLGSQLEATLYSDQELSYIQQGEE-AM$_{OX}$QK. Nearly the complete b- and y-series are visible. The typical generation of dehydroalanine from phosphoserine, resulting in the loss of −98 Da (−H$_3$PO$_4$) occurs from b$_5$ excluding Ser[60], Ser[65], Thr[68], and Ser[73]. *B*, proposed model of StAR phosphorylation by cGMP- and cAMP-dependent protein kinase. As discussed in the text, both kinases show different specificities for the various identified phosphorylation sites in StAR. A conclusion for Ser[55] and/or Ser[56] was not possible on the basis of the acquired data.

a previously reported inhibitory effect of cGMP on ACTH-stimulated aldosterone production. This dual effect of cGMP is mediated by different target enzymes, *i.e.* PKG II mediates aldosterone production, whereas PDE II degrades ACTH-stimulated cAMP and inhibits aldosterone production. These effects of cGMP add another facet to the fine-tuning of the renin-angiotensin-aldosterone system, which regulates fluid volume and blood pressure. PKG II maintains basal aldosterone activity in the absence of acute stimulators like ACTH, and phos-

phorylates StAR, which controls the rate-limiting step in aldosterone synthesis. Although PKA increases both StAR phosphorylation and gene expression, PKG II only phosphorylates StAR (also see below). In any case, the low level stimulation of aldosterone production caused by cGMP would be more suited to maintain basal aldosterone activity than would the large amount of aldosterone production evoked by ACTH at times of stress or exertion.

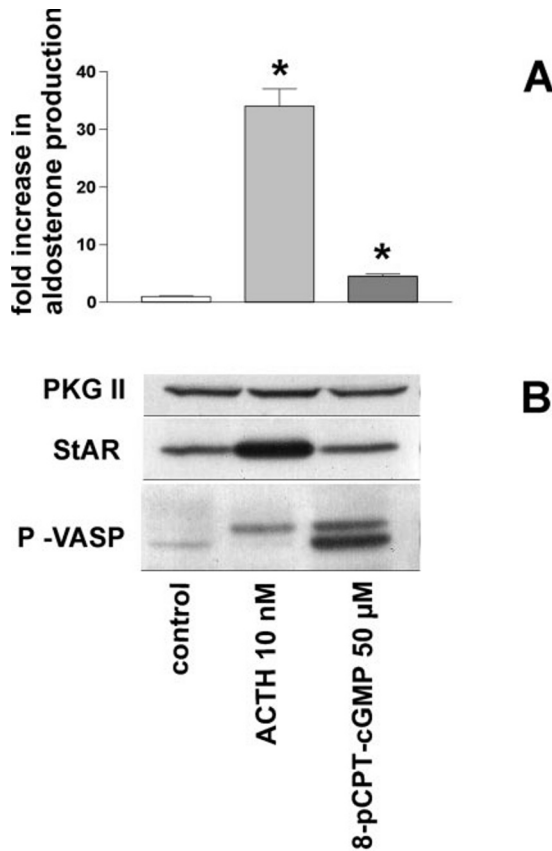The stimulatory effects of cGMP on aldosterone production

FIG. 8. **Activation of PKG II in cultured rat ZG cells stimulates aldosterone production without activating StAR gene expression.** *A*, after 24-h stimulation with the agents shown, incubation medium was analyzed by aldosterone RIA. *B*, cell lysates were processed for Western blotting with PKG II, StAR, or phospho-VASP antibodies. ACTH and 8-pCPT-cGMP significantly enhanced aldosterone production (34 ± 5-fold and 4.8 ± 0.6-fold, respectively) (*A*), whereas StAR protein expression was strongly up-regulated only by ACTH (*B*), although ACTH and 8-pCPT-cGMP activated PKA and PKG, respectively, as demonstrated by VASP phosphorylation. Results are mean $\pm$ S.E. of three different experiments. *, significantly different ($p < 0.05$) from control values.

were initially revealed in our experiments using 8-pCPT-cGMP and Rp-8-pCPT-cGMPS, analogs which selectively activate and inhibit PKG, respectively, without affecting phosphodiesterases. cGMP itself, elevated by atrial natriuretic peptide or NO, could not be used in our system to evaluate the PKG II function in ZG cells, due to its well known inhibitory effect via activation of PDE II (9, 10). For freshly isolated ZG cells, high concentrations of cGMP analogs and sGC inhibitor ODQ (Fig. 3, *A–C*) were used, a phenomenon already known for ZG cells (9), whereas the activation of PKG II in cultured cells was achieved by 10-fold lower concentration of 8-pCPT-cGMP (Figs. 4 and 6).

In contrast to renin-secreting juxtaglomerular cells, where PKG II activation has an opposite (inhibitory) function to PKA activation (15, 31–33), in ZG cells PKG II and PKA act synergistically, both activating aldosterone production. It is known that in other systems high concentrations of cGMP or cGMP analogs may cross-activate PKA (34); therefore, one of the critical goals of our study was to discriminate PKA and PKG II activities in ZG cells. Three series of experiments confirmed that PKG II activation had an independent stimulatory effect on aldosterone production (Figs. 3*C*, 4, and 5). First, we activated PKG II in ZG cells when aldosterone production was already activated by cAMP (ACTH and forskolin) or Ang II. The doses used for activation of PKA (10 nM for ACTH and 10 μM for

forskolin) were already maximal (23),[2] therefore, it is unlikely that under these conditions 8-pCPT-cGMP would further activate PKA. Second, cGMP analogs used in the same concentrations (maximum 500 μM) had no effect on aldosterone release from NCI cells, the cell line that does not express PKG II (Fig. 4). Third, a more direct demonstration of PKG II stimulatory effect was proved by adenoviral gene transfer of PKG II into cultured ZG cells (Fig. 5). Overexpression of PKG II and PKG II K482A had no significant effect on forskolin-stimulated aldosterone production, indicating that the cAMP pathway was not affected by adenoviral infection. Basal aldosterone production was activated in the cells overexpressing wild type PKG II already in the absence of 8-pCPT-cGMP (1.9 ± 0.3-fold, $p < 0.05$ *versus* control), and this activation further dose-dependently increased (Fig. 5). Low doses of 8-pCPT-cGMP, which will not cross-activate PKA, and inhibitory effects of overexpressed the PKG II K482A catalytically inactive mutant, which had no effect on PKA-stimulated aldosterone production, clearly indicate that the stimulatory effects of PKG II on aldosterone production from ZG cells are independent from PKA activity. Two possible mechanisms might explain the dominant negative effect of the catalytically inactive mutant PKG II-K482A on endogenous PKG II. First, the PKG II-K482A mutant might compete with wild type PKG II for substrate binding, and second, because overexpressed PKG II-K482A is still able to bind cGMP, it might reduce the cellular concentration of cGMP or the cGMP analog. A similar effect has been observed in fibroblasts overexpressing a catalytically inactive mutant of PKG I (35) and overexpressing a naturally inactive splice variant of PKG II (36). The inhibitory effect of the mutant on wild type PKGs could be abolished by raising the concentration of 8-pCPT-cGMP, suggesting that the inactive mutant competed with wild type PKGs for the cGMP analog.

The last series of experiments was designed to elucidate the molecular mechanisms of PKG II-dependent activation of aldosterone production. Because the activation of steroidogenesis is directly correlated with StAR protein expression and phosphorylation (3, 24), we focused on PKG II-specific StAR phosphorylation and StAR gene expression. It is generally accepted that the activity of StAR protein directly correlates with its phosphorylation, however, the information about the kinases that can phosphorylate StAR is limited to PKC (phosphorylation sites are not yet identified (37)) and PKA, which phosphorylate StAR protein at least at two residues (serines 56 and 195, numbers are given for the human StAR sequence), and phosphorylation at serine 195 resulted in an increase of the StAR protein activity (3). In our experiments StAR protein was phosphorylated *in vitro* by PKG II and PKA (Figs. 6 and 7). Analysis of the phosphorylation sites revealed, in addition to already known sites at serine 55/56 and 195, a new site at serine 276 for PKA. Serine 55 and/or 56 (but not serine 195) were also phosphorylated by PKG II (Fig. 7 *B*). In addition PKG II phosphorylated serine 99, a conserved serine among human, rat, mouse, porcine, and bovine StAR sequences. With its two basic lysine residues amino-terminal to the phosphorylated serine, this site forms a selective determinant for PKG over PKA (38). Probably, an increase of aldosterone synthesis by activation of PKG II is mediated by phosphorylation of StAR at serine 99; however, further experiments are needed to characterize the functional consequences of PKG II-induced StAR phosphorylation for steroidogenesis.

StAR gene expression is up-regulated by almost all factors that are activating steroidogenesis, including ACTH, calcium

and potassium ions, Ang II, and other factors (reviewed in Ref. 1). Although the StAR gene lacks cAMP-responsible element (CRE) recognition sites in its promoter region (39), cAMP is one of the most potent activators of the StAR promoter, probably acting through CCAT/enhancer binding protein (C/EBP) response elements (39–41). Induction of the StAR protein expression also directly correlates with inhibition of the dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on the X-chromosome, gene *1* (DAX-1) and activation of steroidogenic factor 1 (SF-1) transcription factors (42). However, in our experiments, PKG II activated aldosterone release from ZG cells but, in contrast to PKA, did not stimulate StAR gene expression (Fig. 8), giving additional proof that different mechanisms are involved in regulating steroidogenesis by these two protein kinases.

In summary, we present here a novel mechanism for regulating aldosterone production by PKG II, which is important for maintaining the basal level of circulating aldosterone. Enhanced aldosterone release by activated PKG II directly correlated with the phosphorylation of StAR protein, however, in contrast to other known activators of aldosterone system, PKG II did not activate StAR protein gene expression.

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