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RGS2 Is Regulated by Angiotensin II and Functions as a Negative Feedback of Aldosterone Production in H295R Human Adrenocortical Cells

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Regulator of G protein signaling (RGS) proteins interact with Gα-subunits of heterotrimeric G proteins, accelerating the rate of GTP hydrolysis and finalizing the intracellular signaling triggered by the G protein-coupled receptor-ligand interaction. Angiotensin (Ang) II interacts with its G protein-coupled receptor in zona glomerulosa adrenal cells and triggers a cascade of intracellular signals that regulates steroidogenesis and proliferation. We studied Ang II-mediated regulation of RGS2, the role of RGS2 in steroidogenesis, and the intracellular signal events involved in H295R human adrenal cells. We report that both H295R cells and human adrenal gland express RGS2 mRNA. In H295R cells, Ang II caused a rapid and transient increase in RGS2 mRNA levels quantified by real-time RT-PCR. Ang II effects were mimicked by calcium ionophore A23187 and blocked by calcium channel blocker nifedipine. Ang II effects also were blocked by calmodulin antagonists (W-7 and calmidazolium) and calcium/calmodulin-dependent kinase antagonist KN-93. RGS2 overexpression by retroviral infection in H295R cells caused a decrease in Ang II-stimulated aldosterone secretion but did not modify cortisol secretion. In reporter assays, RGS2 decreased Ang II-mediated aldosterone synthase up-regulation. These results suggest that Ang II up-regulates RGS2 mRNA by the calcium/calmodulin-dependent kinase pathway in H295R cells. RGS2 overexpression specifically decreases aldosterone secretion through a decrease in Ang II-mediated aldosterone synthase-induced expression. In conclusion, RGS2 expression is induced by Ang II to terminate the intracellular signaling cascade generated by Ang II. RGS2 alterations in expression levels or functionality could be implicated in deregulations of Ang II signaling and abnormal aldosterone secretion by the adrenal gland. (*Endocrinology* 147: 3889–3897, 2006)

REGULATOR OF G PROTEIN signaling (RGS) proteins comprise a complex and diverse family of proteins that interact with activated Gα-subunits of heterotrimeric G proteins accelerating GTP hydrolysis and consequently Gα-inactivation and termination of G protein signaling (1–5). G protein signaling pathways are essential for adrenal gland regulation. Angiotensin (Ang) II, ACTH, and endothelin (ET)-1 are among the most potent physiological inducers of adrenal steroidogenesis. These peptide hormones regulate adrenal cells through G protein-mediated intracellular signaling pathways. When the ligand binds its seven-transmembrane G protein-coupled receptor (GPCR), it causes a conformational change of the receptor that promotes the exchange of GDP by GTP on the Gα-subunit of G proteins. G proteins are heterotrimeric proteins composed of Gα-, Gβ-, and Gγ-polypeptides. When G protein binds GTP, it becomes activated, and the Gα-subunit dissociates from the Gβγ-complex. Both the activated Gα and the Gβγ-complex interact with effector molecules to generate the intracellular signaling events triggered by the ligand. Termination of signaling depends on the rate of hydrolysis of the GTP bound to the Gα-protein. Gα has intrinsic GTPase activity, but its hydrolysis rate is too slow to account for the rapid termination of the intracellular signaling observed in physiological processes.

The more than 20 members of the RGS family of polypeptides are characterized by the presence of a highly conserved approximately 130-amino acid RGS domain to which the Gα-subunit of heterotrimeric G proteins is bound. RGS2 is a member of the B/4R subfamily within the RGS protein family that is characterized by the presence of an amphipathic helix domain in addition to the RGS domain common to all family members. RGS2 mainly interacts with $G\alpha_{q/11}$ (6–8), which is the Gα-subclass that has been implicated in Ang II receptor signal transduction (9).

Several aspects of RGS2 physiology have been elucidated in studies of the RGS2 knockout mouse. The RGS2 *null* mouse has defects in several systems, including reduced T cell proliferation and IL-2 production, increased anxiety response, decreased male aggression, decreased density of apical and basilar spines in hippocampal CA1 neurons, and Ang II-dependent hypertension (10). RGS2 knockout mice present a strong hypertensive phenotype that is mostly dependent on Ang II stimulation because AT1 receptor antagonist treatment normalizes blood pressure (11). RGS2 knockout hy-

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Abbreviations: Ang, Angiotensin; CaMK, calcium/calmodulin-dependent kinase; ET, endothelin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein-coupled receptor; PK, protein kinase; PMA, phorbol 12-myristate 13-acetate; RAS, renin Ang system; RGS, regulator of G protein signaling; SKF, SKF-96365; VSMC, vascular smooth muscle cell.

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pertension is associated with renovascular abnormalities, persistent constriction of the resistant vasculature, and prolonged response of the vasculature to vasoconstrictors *in vivo* (11).

RGS2 expression is highly regulated both *in vivo* and *in vitro*. Its expression in the brain is regulated by physiological and pharmacological agents. Stimuli that evoke neuronal plasticity, including electroconvulsive seizures, cause a rapid and transient increase in RGS2 mRNA in neurons of the hippocampus, cortex, amygdala, and striatum (12). Single injections of cocaine, amphetamine, or methamphetamine cause rapid increases in RGS2 mRNA levels in caudate putamen (13, 14). RGS2 is also involved in bone metabolism because systemic administration of parathyroid hormone increases RGS2 mRNA in metaphyseal bone (15). *In vitro*, RGS2 is up-regulated by several stimuli, including concanavalin A in human blood mononuclear cells (16), forskolin in rat pheochromocytoma PC12 cells (17), glucose-dependent insulinotropic polypeptide in murine pancreatic β-TC3 cells (18), prostaglandins E1 and E2 in human U937 monocyte cells (19), and muscarinic receptor activation by carbachol or heat shock in human neuroblastoma SH-SY5Y cells (20, 21). Ang II also up-regulates RGS2 mRNA in human vascular smooth muscle cells (VSMCs) (22), parathyroid hormone in rat diaphyseal or metaphyseal osteoblasts cells (15), muscarinic or β-adrenergic receptor activation in human astrocytoma 1321N1 cells (23), and oxytocin in human myometrial cells (24). The intracellular signaling mechanisms that mediate RGS2 up-regulation are system-dependent and have been reported to include the cAMP/adenylyl cyclase/protein kinase (PK) A (15, 17, 23, 25, 26), calcium (16, 24), and PKC (20–22, 24) pathways.

Although the stimulatory effect of Ang II upon aldosterone secretion by adrenal gland has been studied for decades, much remains to be elucidated about signaling termination. Because Ang II receptors generate a series of intracellular signaling events by interacting mainly with the $G\alpha_{q/11}$-subclass of G protein, and RGS2 mainly interacts with the same subclass of heterotrimeric G proteins, we hypothesize that RGS2 is up-regulated by Ang II in adrenal cells and functions as a negative feedback mechanism on Ang II receptor signaling. Human H295R adrenocortical cells were used as the experimental model because it is the only human adrenal cell line that expresses all the steroidogenic enzymes and has a steroid secretion pattern and regulation similar to primary cultures of adrenal cells (27, 28). In the present study, we examined RGS2 expression, its regulation in H295R human adrenocortical cells, and the intracellular signaling mechanisms by which Ang II mediates RGS2 up-regulation. In addition, we tested whether RGS2 protein overexpression alters aldosterone secretion by adrenal cells.

Materials and Methods

Materials

Ang II and ET-1 were obtained from Sigma Chemical Co (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA), forskolin, A23187, nifedipine, SKF-96365 (SKF), calmidazolium, KN-93, neomycin, bisindolylmaleimide I (GF 109203X), bisindolylmaleimide V, calphostin C, chelerythrine, Gö 6976, Gö 6983, Ro-31-8220, Ro-32-0432, and myristoylated PKC inhibitor peptide (20–28) were obtained from EMD Biosciences (San Diego, CA). W-7 was obtained from Tocris (Ellisville, MO). Human adrenal total RNA was obtained from several sources: hAd1 (59-yr-old male donor) from BioChain Institute, Inc. (Hayward, CA), hAd2 (pooled from 61 male/female Caucasians donors, ages 15–61) from BD Biosciences (Mountain View, CA), and hAd3 (30-yr-old female donor) from Stratagene (La Jolla, CA). RGS2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA; catalog no. H-90).

Cell culture

H295R human adrenocortical cells (29) (a generous gift from Dr. W.E. Rainey, University of Texas Southwestern, Dallas, TX) were cultured in H295R complete media containing DMEM:F12 (1:1) supplemented with 2% Ultroser G (Biosepra, Villeneuve-la-Garenne, France), ITS-Plus (Discovery Labware, Bedford, MA), and antibiotic/antimycotic mixture (Invitrogen, Carlsbad, CA) as we previously described (30) until subconfluence in six-well plates. Media were replaced with 3 ml fresh media containing different agents and cultured for 3 h more unless otherwise indicated. At the end of the incubation period, media were removed and saved for steroid determination. Inhibitors were added 30 min before other reagents.

RNA extraction and RT-PCR

Total RNA was extracted with the RNeasy Micro kit (QIAGEN, Valencia, CA) and on-column deoxyribonuclease digested. For RT, 5 μg total RNA was incubated with 0.5 μg T_{12}VN and Superscript III (Invitrogen) following the manufacturer's suggested protocol. RGS2 primers (forward, 5′-GTTGGGTAGTGAATCAGGAAGC-3′; reverse, 5′-GACCACCTATTCCCTTCTTGC-3′) were designed with Primer3 software (31). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were previously described (30). Real-time PCR was performed with 1 μl RT product, 1 μl Titanium *Taq* DNA polymerase (CLONTECH, Mountain View, CA), 1:20,000 dilution SYBR Green I (Molecular Probes, Carlsbad, CA), 0.2 mM dNTPs, and 0.1 μM each primer. Cycling conditions were 1 min at 95 C, 50 cycles of 15 sec at 95 C, 15 sec at 60 C, and 1 min at 72 C. Real-time data were obtained during the extension phase, and threshold cycle values were obtained at the log phase of each gene amplification. PCR product quantification was performed by the relative quantification method (32) and standardized against GAPDH. Efficiency for each primer pair was assessed by using serial dilutions of RT product. Results are expressed as arbitrary units and normalized against GAPDH mRNA expression.

Plasmids

Plasmids containing the human RGS2 insert in pcDNA3.1 and pDNR-1r were obtained from the University of Missouri at Rolla cDNA Resource Center (http://www.cdna.org). RGS2 was transferred from pDNR-1r to the retroviral BD Creator acceptor vector pLP-LNCX using the manufacturer's suggested protocol. Reporters plasmids carrying the human CYP11B1 and CYP11B2 promoters (33) (generously provided by Dr. W. E. Rainey) were generated transferring the promoter regions from pGL3-Basic to pGL4.10[luc2] (Promega, Madison, WI). pSEAP2-Control and pVSV-G plasmids were from CLONTECH.

Reporters assays

H295R cells were grown in 24-well plates with H295R complete media without antibiotic/antimycotics until 90–95% confluent. Cells were transfected with 3 μg DNA/well (1.7 μg reporter plasmid, 0.3 μg pSEAP2-Control, and 1.0 μg pcDNA3.1 or pcDNA3.1-RGS2) and 2 μl/well Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Cells were cultured overnight, media replaced with 0.5 ml/well fresh media, and cultured for an additional 24 h. Media were collected to measure secreted alkaline phosphatase using the Great EscAPe SEAP chemiluminescence kit (CLONTECH). Cells were lysed with Glo Lysis buffer (Promega), and luciferase activity was quantified with the Bright-Glo Luciferase assay kit (Promega).

Retroviral production and infection

To generate VSV-G pseudotyped retroviral particles, GP2–293 cells (CLONTECH) were cultured in DMEM supplemented with 10% fetal

bovine serum until 60–70% confluent. Cells were transfected with retroviral vector and pVSV-G (0.25 $\mu g/cm^2$, 1:1 molar ratio) using Trans-IT 293 transfection reagent (2 $\mu l/\mu g$ DNA, Mirus Bio, Madison, WI). Media were replaced after 4 h, and cells were cultured for an additional 48 h. Cell culture supernatant was removed, centrifuged for 5 min at 200 × *g* at 4 C, filtered through a 0.45-μm PES membrane, aliquoted, and stored at −80 C. For retroviral infection, confluent H295R cells were split 1:3 in six-well plates and cultured overnight. Retroviral supernatant was diluted 1:1 with fresh media, added to cells, and cultured for 24 h. Media were replaced, and cells were selected with 500 μg/ml G-418. Infection efficiency was more than 80% of the cell population. After antibiotic selection, cells were cultured for at least 4 wk in the absence of selecting agent before performing the experiments to avoid any confounding effect due to the selecting antibiotic.

Steroid ELISA

Aldosterone and cortisol were measured in cell culture supernatant by ELISA using monoclonal or polyclonal antibodies, respectively, as we previously described (34, 35). Assay sensitivity was 20 pg/ml for aldosterone and 1 ng/ml for cortisol.

Western blot

Cell cultures were lysed with M-PER buffer (Pierce, Rockford, IL) containing 5 mM EDTA, 1 mm EGTA, protease inhibitor cocktail (Roche, Indianapolis, IN), and phosphatase inhibitors cocktails I and II (EMD Biosciences). Cell extracts were separated by 15% PAGE and blotted on polyvinylidene difluoride membranes using standard techniques.

Statistical analysis

All results were expressed as mean ± SEM. Two groups were compared by Student's *t* test, and multiple groups were analyzed by one-way ANOVA followed by Tukey's *post hoc* comparisons. Time-response curves were tested by two-way ANOVA followed by Bonferroni comparisons. Dose-response curves were adjusted to a four-parameter sigmoidal equation, and its parameters were tested by F test; values were tested by two-way ANOVA followed by Bonferroni comparisons. Differences were considered statistically significant at $P < 0.05$. Statistical calculations were performed with GraphPad Prism package version 4.03 (GraphPad Software, Inc., San Diego, CA).

Results

H295R cells and human adrenal gland express RGS2

Human adrenal gland and H295R human adrenocortical cells express RGS2 mRNA. Figure 1 shows an agarose gel electrophoresis of RGS2 PCR products with RNA from adrenal glands of several different sources and H295R cells as template. The minus RT controls show no amplification. GAPDH was used as the housekeeping control gene.

RGS2 is up-regulated by Ang II

H295R human adrenocortical cells were incubated with 100 nM Ang II for increasing time periods up to 72 h, and RGS2 mRNA expression was quantified by real-time PCR (Fig. 2A). Ang II caused a 4-fold increase in RGS2 mRNA levels 3–6 h after the initiation of hormone stimulation. RGS2 mRNA then declined to basal levels within 12 h, decreased to under control cell levels, and remained suppressed for 72 h after the initiation of Ang II treatment. When H295R cells were treated with Ang II for shorter time periods, it was observed that RGS2 mRNA levels reach almost maximal stimulation after only 1 h treatment (Fig. 2B). To study the effect of different doses of Ang II and other agents on RGS2 mRNA expression, H295R cells were incubated with increasing concentrations of Ang II and other aldosterone secretagogues for 3 h, and RGS2 mRNA was quantified (Fig. 3). Ang II (0.1–100 nM) dose-dependently increased RGS2 mRNA levels. Potassium (16 mM) caused a 2-fold increase in RGS2 mRNA (1.98 ± 0.06 *vs.* 1.00 ± 0.07, $P < 0.01$). On the other hand, ET-1 (10 nM) or forskolin (10 μM) did not modify RGS2 mRNA levels. Ang II intracellular signaling in adrenal cells is known to be mainly mediated by PKC and the calcium/calmodulin-dependent kinase (CaMK) pathways (9, 36–38). Experiments were done to determine whether either of these pathways is involved in the regulation of RGS2 mRNA expression.

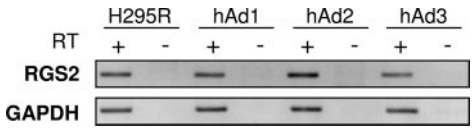

FIG. 1. Expression of RGS2 mRNA in H295R cells and human adrenal gland detected by agarose gel electrophoresis of RT-PCR in the presence (+) or absence (−) of reverse transcriptase. GAPDH mRNA expression was used as control. hAd1, 59-yr-old male donor; hAd2, pooled from 61 male/female white donors, ages 15–61; hAd3, 30-yr-old female donor.

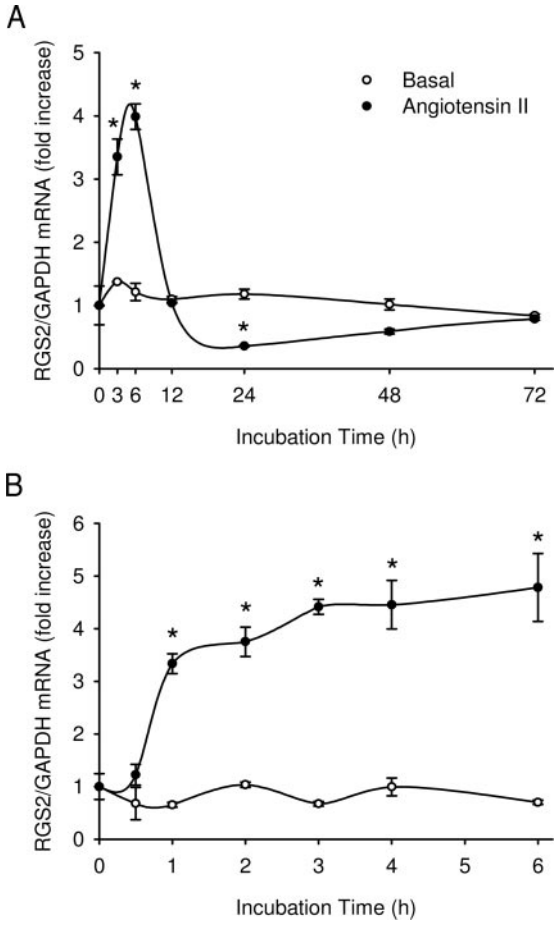

FIG. 2. Ang II up-regulates RGS2 mRNA. H295R cells were incubated with or without 100 nM Ang II for different time points, then RNA was extracted, and RNA was quantified by real-time RT-PCR. RGS2 mRNA was normalized by GAPDH mRNA expression and expressed as fold increase *vs.* time = 0. *, $P < 0.01$ *vs.* basal.

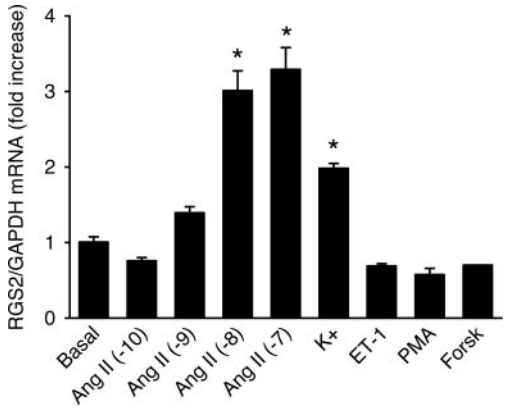

FIG. 3. Effect of different agents on RGS2 mRNA expression. H295R cells were incubated with increasing concentrations of Ang II, potassium (K+; 16 mM), ET-1 (10 nM), PMA (10 nM), or forskolin (Forsk; 10 μM) for 3 h, RNA was extracted, and then RNA was quantified by real-time RT-PCR. RGS2 mRNA was normalized by GAPDH mRNA expression and expressed as fold increase *vs.* control incubations (basal). *, $P < 0.01$ *vs.* basal.

PKC is not involved in Ang II-mediated RGS2 up-regulation

Incubation of H295R cells with a PKC activator, PMA (10 nM), for 3 h did not modify RGS2 mRNA levels (Fig. 3). To determine the effect of PKC inhibitors on Ang II-mediated RGS2 regulation, H295R cells were preincubated with several PKC inhibitors that specifically inhibit different PKC isoforms for 30 min before stimulation with Ang II (100 nM) for 3 h (Fig. 4). Although some PKC inhibitors show a tendency to decrease Ang II-mediated RGS2 mRNA levels, none of them reach significance. To further study the possible role of PKC in RGS2 mRNA regulation, H295R cells were pretreated with a high dose of the PKC activator PMA (10 μM) for 24 h to down-regulate PKC before stimulation with Ang II (100 nM) for 3 h (Fig. 5). Although extended treatment with high-dose PMA caused an increase in RGS2 mRNA levels (1.00 ± 0.03 *vs.* 1.95 ± 0.08, $P < 0.01$), subsequent treatment with Ang II up-regulated RGS2 mRNA to levels similar to control cells pretreated with vehicle (DMSO) (3.24- *vs.* 3.25-fold increase Ang II/basal, not significant).

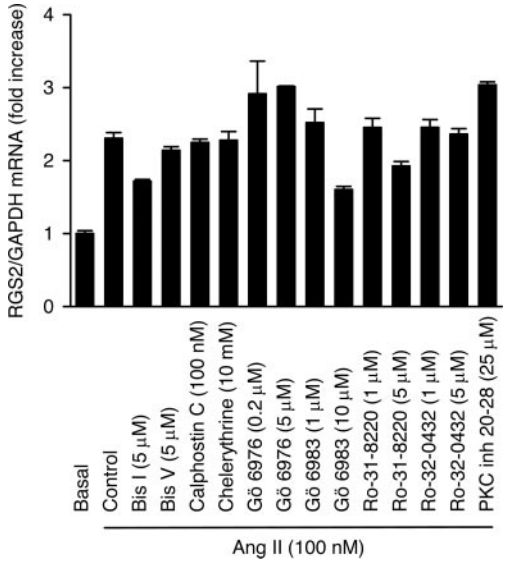

FIG. 4. Effect of PKC inhibitors on RGS2 mRNA expression. H295R cells were treated with different PKC inhibitors or vehicle for 30 min and then incubated in the presence or absence of Ang II (100 nM) for 3 h. RNA was extracted and then quantified by real-time RT-PCR. RGS2 mRNA was normalized by GAPDH mRNA expression and expressed as fold increase *vs.* basal.

Calcium signaling is involved in Ang II-mediated RGS2 up-regulation

To examine the role of the calcium signaling pathway in Ang II-mediated RGS2 regulation, H295R cells were incubated with the ionophore A23187 (10 μM) for 3 h. A23187 caused a 1.7-fold increase in RGS2 mRNA compared with control cells (1.00 ± 0.04 *vs.* 1.71 ± 0.09, $P < 0.01$).

To analyze whether calcium channel blocking would alter Ang II- or potassium-mediated RGS2 up-regulation, H295R cells were preincubated with the L-type Ca^{2+} channel-selective blocker nifedipine (10 μM) for 30 min before stimulation with Ang II (100 nM) or potassium (16 mM) for 3 h (Fig. 6A). Nifedipine decreased both Ang II (3.05 ± 0.65 *vs.* 1.88 ± 0.02, $P < 0.01$)- and potassium (2.16 ± 0.07 *vs.* 0.89 ± 0.06, $P < 0.01$)-mediated RGS2 mRNA up-regulation.

SKF, an inhibitor of both receptor-mediated and voltage-gated calcium influx entry (39), was used to verify the involvement of calcium signaling on RGS2 mRNA up-regulation. H295R cells were preincubated with SKF (25 μM) for 30 min and then stimulated with Ang II (100 nM) or potassium (16 mM) for 3 h (Fig. 6B). SKF blocked both Ang II (2.22 ± 0.20 *vs.* 1.38 ± 0.05, $P < 0.01$)- and potassium-mediated (1.63 ± 0.07 *vs.* 0.79 ± 0.01, $P < 0.01$) RGS2 mRNA up-regulation.

Calmodulin/CaMK mediates RGS2 mRNA up-regulation by Ang II

Calcium mediates most of its effects through binding to the calcium binding protein calmodulin, causing a conformational change that allows calmodulin to interact with and modify the activity of its target proteins. We study the effect of two calmodulin antagonists, W-7 and calmidazolium, on

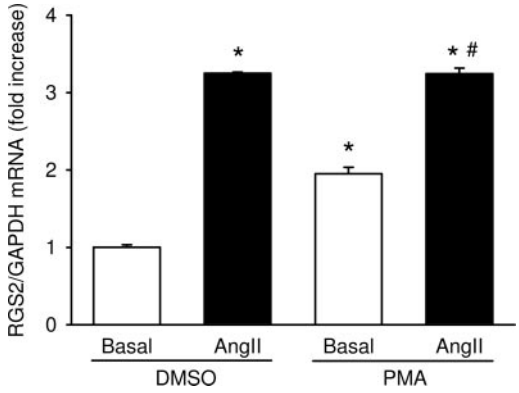

FIG. 5. Effect of PKC down-regulation on RGS2 mRNA expression. H295R cells were treated with 10 μM PMA or vehicle (DMSO) for 24 h and then incubated in the presence or absence of 100 nM Ang II for 3 h. RNA was extracted and then quantified by real-time RT-PCR. RGS2 mRNA was normalized by GAPDH mRNA expression and expressed as fold increase *vs.* DMSO-basal incubations. *, $P < 0.01$ *vs.* DMSO-basal; #, $P < 0.01$ *vs.* PMA-basal.

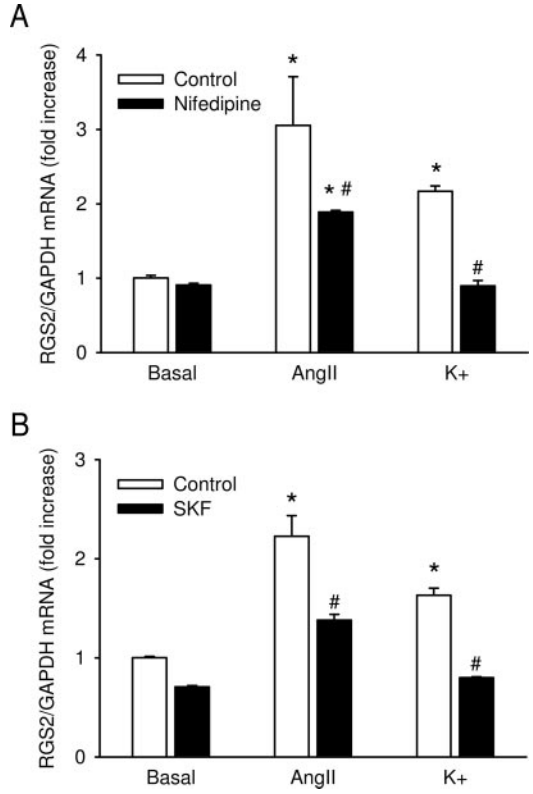

FIG. 6. Effect of calcium channel blockers nifedipine and SKF on RGS2 expression. H295R cells were treated with 10 μM nifedipine (A), 25 μM SKF (B), or vehicle for 30 min and then incubated in the presence or absence of Ang II (100 nM) or potassium (K+; 16 mM) for 3 h. RNA was extracted and then quantified by real-time RT-PCR. RGS2 mRNA was normalized by GAPDH mRNA expression and expressed as fold increase *vs.* control. *, $P < 0.01$ *vs.* basal-control; #, $P < 0.01$ *vs.* control for its treatment.

basal and stimulated conditions. H295R cells were preincubated with W-7 (50 μM) or calmidazolium (10 μM) for 30 min and then treated with Ang II (100 nM) or potassium (16 mM) for 3 h. W-7 caused a 40% decrease in Ang II-mediated RGS2 mRNA induction (2.50 ± 0.05 *vs.* 1.90 ± 0.02, $P < 0.001$) and a 62% decrease in potassium-mediated RGS2 induction (1.80 ± 0.12 *vs.* 1.30 ± 0.07, $P < 0.01$) (Fig. 7A). Similar experiments were performed with the calmodulin antagonist calmidazolium. Calmidazolium caused a 46% decrease in Ang II-mediated RGS2 mRNA induction (2.50 ± 0.05 *vs.* 1.80 ± 0.07, $P < 0.001$) and completely blocked potassium-mediated RGS2 mRNA induction (1.80 ± 0.12 *vs.* 0.84 ± 0.02, $P < 0.01$), reaching values indistinguishable from unstimulated cells treated with calmidazolium (Fig. 7B).

Once complexed with calcium, calmodulin can interact with CaMK, allowing phosphorylation of the inhibitory subunit by the CaMK regulatory subunit and activating the PK. Several CaMK isoforms are expressed in the adrenal gland and are some of the main effectors of calcium intracellular signaling in this gland. To analyze whether RGS2 mRNA induction is mediated by CaMK, H295R cells were pretreated with the specific inhibitor of CaMK I, II, and IV, KN-93 (5 μM), for 30 min and then incubated in the presence or absence of Ang II (100 nM) or potassium (16 mM). KN-93 caused a 47% decrease in Ang II-mediated RGS2 mRNA induction (2.70 ± 0.05 *vs.* 1.90 ± 0.08, $P < 0.001$) and completely blocked potassium-mediated RGS2 mRNA induction (1.70 ± 0.09 *vs.* 0.84 ± 0.01, $P < 0.001$), reaching values indistinguishable from unstimulated cells treated with KN-93 (Fig. 7C).

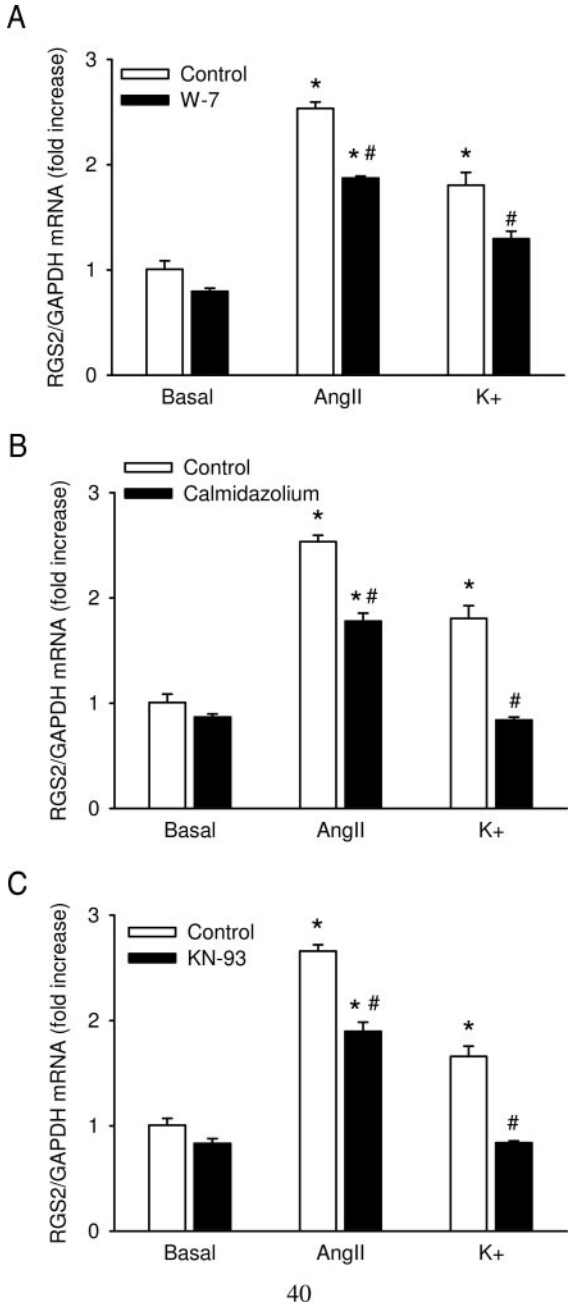

FIG. 7. Effect of calmodulin and CaMK inhibitors on RGS2 expression. H295R cells were treated with 50 μM W-7 (A), 10 μM calmidazolium (B), 5 μM KN-93 (C), or vehicle for 30 min and then incubated in the presence or absence of Ang II (100 nM) or potassium (K+; 16 mM) for 3 h. RNA was extracted and then quantified by real-time RT-PCR. RGS2 mRNA was normalized by GAPDH mRNA expression and expressed as fold increase *vs.* control. *, $P < 0.01$ *vs.* basal-control; #, $P < 0.01$ *vs.* control for its treatment.

RGS2 overexpression decreases aldosterone secretion

To examine the global effect of RGS2 up-regulation on steroid secretion, we generated stably RGS2-overexpressing

cell lines in H295R cells. H295R cells infected with a retrovirus expressing RGS2 under control of the cytomegalovirus promoter (H295R-RGS2 cells) show increased levels of both RGS2 mRNA and protein (Fig. 8, C and D). To study whether RGS2 overexpression alters the steroid secretion response to Ang II, H295R and H295R-RGS2 cells were incubated with increasing concentrations of Ang II, and cell culture supernatants were assayed for aldosterone and cortisol. RGS2 overexpression caused a significant decrease in aldosterone secretion at all doses of Ang II tested (Fig. 8A). RGS2 overexpression reduced basal (22.3 ± 4.6 *vs.* 3.6 ± 0.6 pg aldosterone/μg protein·d, $P < 0.001$) and maximal stimulation (108.4 ± 6.7 *vs.* 29.6 ± 1.1 pg aldosterone/μg protein·d, $P < 0.001$) aldosterone secretion. RGS2 overexpression did not modify Ang II dose-response pEC_{50} (−8.96 ± 0.23 *vs.* −8.62 ± 0.11 $\log_{10}$ M, not significant), nor did it modify basal and maximally stimulated levels or pEC_{50} for cortisol secretion (Fig. 8B).

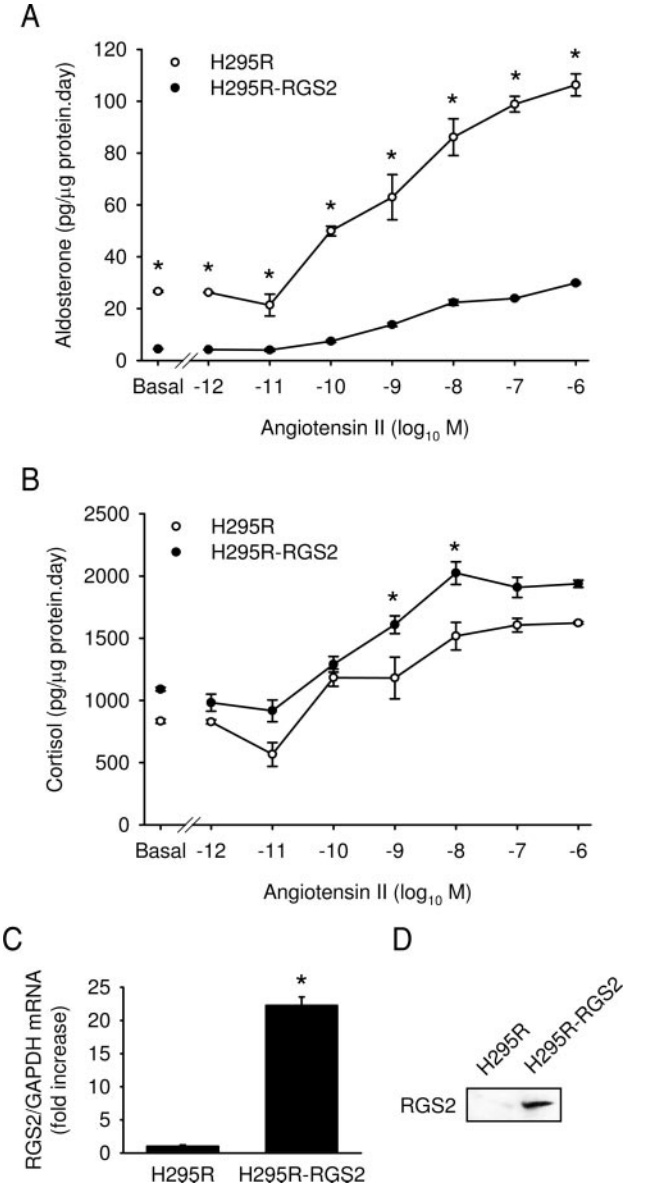

FIG. 8. Effect of RGS2 overexpression on aldosterone and cortisol secretion. H295R cells were infected with retroviruses expressing RGS2, selected, and incubated with increasing concentrations of Ang II for 24 h. Aldosterone (A) and cortisol (B) concentration was measured in media supernatant and cells lysed to quantify total protein. RGS2 mRNA levels (C) were measured by real-time RT-PCR. RGS protein levels (D) were detected by Western blot. *, $P < 0.01$ *vs.* control cells.

RGS2 and steroidogenic pathway gene expression

Two key regulatory enzymes in adrenocortical steroid biosynthesis are 11β-hydroxylase (CYP11B1 gene) and aldosterone synthase (CYP11B2 gene), which catalyze the last metabolic steps in glucocorticoid and mineralocorticoid pathways, respectively (40). To study the effect of RGS2 on gene expression of these two enzymes, we performed cotransfection studies with luciferase reporter plasmids under the control of human CYP11B1 and CYP11B2 promoters and RGS2 under basal and Ang II (100 nM)-stimulated conditions. Ang II treatment of transfected cells caused a minimal but significant increase in CYP11B1 expression that was completely blocked by RGS2 (1.19 ± 0.02 *vs.* 1.03 ± 0.02, $P < 0.01$) (Fig. 9A). Ang II treatment of transfected cells caused a 2.2-fold increase in CYP11B2 expression that was almost completely blocked by RGS2 cotransfection (2.25 ± 0.06 *vs.* 1.26 ± 0.04, $P < 0.01$) (Fig. 9B).

Discussion

In the present study, we demonstrate that: 1) human adrenal gland and H295R cells express RGS2 mRNA; 2) RGS2 mRNA is up-regulated by Ang II in H295R cells; 3) RGS2 mRNA up-regulation involves the calcium signaling pathway, including calcium channels, calmodulin, and CaMK; and 4) RGS2 overexpression causes a decrease in Ang II-mediated aldosterone secretion.

Hormonal signals are crucial for the integration of different organs and systems within living organisms. After binding to cognate receptors, hormones trigger a cascade of intracellular events that cause specific physiological responses. The termination of intracellular signaling events is equally as important as their initiation to achieve homeostasis at the

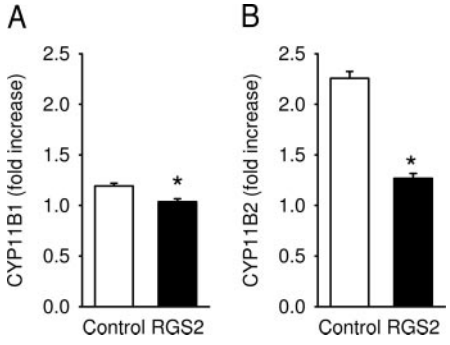

FIG. 9. Effect of RGS2 on 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) reporter expression. H295R cells were transfected with CYP11B1 (A) or CYP11B2 (B) reporter plasmids and RGS2 expressing plasmid or control plasmid (pcDNA3.1), allowed to recover overnight, and incubated in the presence or absence of 100 nM Ang II for 24 h. Data are expressed as Ang II/Basal fold induction. *, $P < 0.01$ *vs.* control.

cellular and whole organism level. Ang II modifies several aspects of adrenal cell metabolism and physiology including steroid secretion and zona glomerulosa cell proliferation. Ang II binds to its GPCRs, which interact with G proteins, that after exchange of GDP by GTP will be activated and available to interact with its target effector proteins to transduce Ang II signal from the extracellular space to the intracellular compartment. The results described in the present report indicate that RGS2 is a key molecule required for Ang II signaling homeostasis in adrenal cells. RGS2 functions as a turn-off signal of Ang II action in adrenal cells, causing a negative feedback on the same receptor that mediates RGS2 up-regulation.

We report that RGS2 mRNA is expressed in the adrenals of both men and women, in accordance with Rainey *et al.* (41), who also have reported that RGS2 mRNA is up-regulated in adults when compared with the fetal human adrenal gland. Ang II rapidly and transiently up-regulates RGS2 mRNA levels in H295R cells. Maximal RGS2 levels were reached within 1 h after hormone addition. Elevated levels of RGS2 mRNA persisted for several hours before returning to basal levels. Similar RGS2 mRNA modulation triggered by different stimuli has been reported in other systems. *In vivo*, RGS2 mRNA in the caudate putamen reaches maximal levels 1 h after amphetamine injection, resembling immediate early regulated genes such as c-*fos* (13). Similar *in vivo* rapid and transient up-regulation of RGS2 mRNA has been observed in the rat brain after stimuli of neuronal plasticity (12) and femoral metaphysis after parathyroid hormone injection (15). *In vitro*, similar rapid and transient up-regulation of RGS2 has been reported in SH-SY5Y neuroblastoma cells stimulated with carbachol (20) or heat shock (21), VSMC treated with Ang II (22, 42), osteoblast cells stimulated with parathyroid hormone (15, 25, 26), and myometrial cells treated with oxytocin (24).

RGS2 levels are regulated by several signaling pathways depending on the cells and system studied, including the cAMP/adenylyl cyclase/PKA, calcium, or PKC pathways. RGS2 mRNA levels in H295R cells were mainly regulated by the calcium/calmodulin/CaMK pathway. This intracellular signaling pathway is also the most important one triggered by Ang II in adrenal cells. In H295R cells, both Ang II and potassium have been reported to rapidly and transiently increase extracellular calcium influx (29, 43, 44). Calcium-mediated signaling pathways have been implicated in RGS2 mRNA regulation in other systems, including cultured human blood mononuclear cells in which RGS2 mRNA levels are increased by ionomycin treatment (16). RGS2 mRNA levels were up-regulated after only 30 min of ionomycin treatment of the mononuclear cells and returned to basal levels 2 h after treatment initiation. Calcium signaling is also involved in the up-regulation of RGS2 levels in cultured human myometrial cells by oxytocin (24). Oxytocin-mediated RGS2 up-regulation was blocked by the calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester and mimicked by the calcium ionophore A23187. In VSMC, Ang II-mediated RGS2 mRNA up-regulation is mediated by PKC and independent of calcium signaling (22). Moreover, RGS2 mRNA up-regulation in VSMC was not modified by the absence of calcium in the media or by the use of intracellular calcium chelator or the calcium ATPase inhibitor thapsigargin (22). These data demonstrate that several signaling pathways regulate RGS2 levels and that different pathways are triggered by the same inducer depending on the cell type.

To study the role of Ang II-mediated RGS2 up-regulation, we overexpressed RGS2 in H295R cells. RGS2 overexpression causes a specific decrease in Ang II-mediated aldosterone production without modifying cortisol secretion. RGS2 decreases basal aldosterone secretion in the absence of added Ang II. This finding is not surprising because H295R cells have an endogenous renin Ang system (RAS) that under basal conditions secretes Ang II, causing basal aldosterone secretion in the absence of exogenous stimuli (45). RGS2 overexpression in H295R cells reduced maximal Ang II-mediated aldosterone secretion but did not modify the responsiveness of the system because it did not modify Ang II pEC_{50} for aldosterone secretion. A similar finding has been reported in HEK cells coexpressing RGS2 and $G\alpha_{q/11}$-coupled muscarinic receptor in which RGS2 overexpression decreased methacholine-mediated inositol 1,4,5-triphosphate production but did not modify the pEC_{50} of the system (46). Studies using RGS2 knockout mice indicate that the lack of RGS2 could alter the magnitude of the output or the sensitivity of the response depending on the system (11, 47, 48). Our results, together with those from other reports, implicate RGS2 in the regulation of both the sensitivity and the magnitude of the response by intracellular signaling systems to hormonal stimulation.

Our results from studies of the H295R-RGS2 cells seem to contradict reports of unmodified aldosterone plasma levels in the RGS2 knockout mouse (49). No plasma Ang II levels or renin activities have been reported in these mice. We speculate that compensatory alterations in the systemic or intraadrenal RAS could account for the normal aldosterone levels observed in these mice under basal metabolic conditions. Although the lack of RGS2 would be expected to increase the duration of the intracellular signaling cascade, persistent secretion of aldosterone would produce physiological effects that would down-regulate the RAS, generating lower levels of circulating and/or locally generated Ang II that would compensate for the intracellular alteration and mask alterations in adrenal aldosterone secretion. If this is indeed what occurs, it would be anticipated that the regulation of aldosterone in these RGS2 knockout mice in response to physiological perturbations, for example, an acute alteration in dietary sodium, would be slower than normal.

Our studies using a reporter system confirm our findings of RGS2 involvement on aldosterone synthesis regulation. These results demonstrate that RGS2 overexpression blunts Ang II-mediated increase in aldosterone synthase expression and indicate that one of the mechanisms by which RGS2 regulates aldosterone production is by directly or indirectly modulating aldosterone synthase expression levels. Aldosterone synthesis is regulated at several points in a series of enzymatically catalyzed reactions starting with cholesterol; however, aldosterone synthase is the only specific and unique enzyme required for aldosterone biosynthesis that is not involved in the synthesis of other adrenal steroid hormones.

In the present report, we show that Ang II up-regulates RGS2 expression and that RGS2 decreases aldosterone secretion by cultured human adrenal cells, blunting the stimulatory effect of Ang II on aldosterone synthesis. These findings elucidate a mechanism by which Ang II intracellular signaling is terminated and Ang II receptor desensitized within the adrenal cell. We speculate that RGS2 is crucial for the regulation of aldosterone synthesis and that alterations in either its expression level or activity due to either a loss or gain of function mutations may lead to abnormal adrenal GPCR signaling. Aldosterone levels are critical not only to salt and water homeostasis but also to multiple other systems. There is ample evidence that aberrant aldosterone levels are responsible for target end organ damage under various pathological conditions; however, the mechanism for this deregulation is not clear (50). Faulty regulation of Ang II receptor signaling and the slow termination of normal hormonal stimulation of aldosterone synthesis may underlie or exacerbate certain renal and cardiovascular diseases.

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D.R., M.P., E.G., L.Y., and C.G. have nothing to declare.

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