Regulators of G-protein signaling 4 in adrenal gland: localization, regulation, and role in aldosterone secretion

Damian G Romero^{1,2}, Ming Yi Zhou³, Licy L Yanes⁴, Maria W Plonczynski¹, Tanganika R Washington^{1,2}, Celso E Gomez-Sanchez^{1,2} and Elise P Gomez-Sanchez^{1,2,5}

¹Division of Endocrinology, G V (Sonny) Montgomery VA Medical Center and ²Department of Medicine, The University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216, USA

³DNA Core, University of Missouri-Columbia, Columbia, Missouri 65211, USA

⁴Departments of Physiology and Biophysics and ⁵Pharmacology and Toxicology, The University of Mississippi Medical Center, Jackson, Mississippi 39216, USA

(Requests for offprints should be addressed to D G Romero; Email: dromero@medicine.umsmed.edu)

Abstract

Regulators of G-protein signaling (RGS proteins) interact with G α subunits of heterotrimeric G-proteins, accelerating the rate of GTP hydrolysis and finalizing the intracellular signaling triggered by the G-protein-coupled receptor (GPCR)–ligand interaction. Angiotensin II (Ang II) interacts with its GPCR in adrenal zona glomerulosa cells and triggers a cascade of intracellular signals that regulates steroidogenesis and proliferation. On screening for adrenal zona glomerulosa-specific genes, we found that RGS4 was exclusively localized in the zona glomerulosa of the rat adrenal cortex. We studied RGS4 expression and regulation in the rat adrenal gland, including the signaling pathways involved, as well as the role of RGS4 in steroidogenesis in human adrenocortical H295R cells. We reported that RGS4 mRNA expression in the rat adrenal gland was restricted to the adrenal zonal glomerulosa and upregulated

by low-salt diet and Ang II infusion in rat adrenal glands *in vivo*. In H295R cells, Ang II caused a rapid and transient increase in RGS4 mRNA levels mediated by the calcium/calmodulin/calmodulin-dependent protein kinase and protein kinase C pathways. RGS4 overexpression by retroviral infection in H295R cells decreased Ang II-stimulated aldosterone secretion. In reporter assays, RGS4 decreased Ang II-mediated aldosterone synthase upregulation. In summary, RGS4 is an adrenal gland zona glomerulosa-specific gene that is upregulated by aldosterone secretagogues, *in vivo* and *in vitro*, and functions as a negative feedback of Ang II-triggered intracellular signaling. Alterations in RGS4 expression levels or functions may be involved in deregulations of Ang II signaling and abnormal aldosterone secretion.

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Introduction

Regulators of G-protein signaling (RGS proteins) comprise a complex and diverse family of proteins that interact with the activated G α subunits of heterotrimeric G-proteins accelerating GTP hydrolysis and consequently G α inactivation and termination of G-protein signaling (Berman & Gilman 1998, De Vries & Gist Farquhar 1999, Burchett 2000, Ross & Wilkie 2000, De Vries *et al.* 2000, Hollinger & Hepler 2002, Abramow-Newerly *et al.* 2006, Sato *et al.* 2006). G-protein signaling pathways are essential for adrenal gland regulation. Peptide hormones angiotensin II (Ang II), adrenocorticotrophic hormone (ACTH), and endothelin-1, potent physiological inducers of adrenal steroidogenesis, regulate adrenal cells through G-protein-mediated intracellular signaling pathways. G-proteins are heterotrimeric proteins composed of G α , G β , and G γ polypeptides. 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 with GTP on the G α subunit of G-proteins. When G-protein binds GTP, it becomes activated and the G α subunit dissociates from the G $\beta\gamma$ complex. Both the activated G α and the G $\beta\gamma$ 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 G α protein. G α 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. RGS-mediated acceleration of the G α -protein intrinsic GTPase activity overcomes this rate-limiting step in GPCR inactivation and signaling termination.

More than 20 members of the RGS family of polypeptides are characterized by the presence of a highly conserved $\sim$ 130 amino acid RGS domain to which the G α subunit of heterotrimeric G-proteins is bound. RGS4 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 (Berman & Gilman 1998, De Vries & Gist Farquhar 1999, Burchett 2000, Ross & Wilkie 2000, De Vries *et al.* 2000, Hollinger & Hepler 2002, Blumer 2005, Abramow--Newerly *et al.* 2006, Sato *et al.* 2006). RGS4 interacts with several G α proteins (Berman *et al.* 1996, Watson *et al.* 1996, Hepler *et al.* 1997, Roy *et al.* 2003), including G $\alpha_{q/11}$ which is the G α subclass that has been implicated in Ang II receptor signal transduction (Spat & Hunyady 2004). RGS4 proteins have been studied in several tissues, including the cardiovascular and nervous systems where it plays very important roles (Riddle *et al.* 2005). In the cardiovascular system, RGS4 levels were reported to be increased in the hearts of pulmonary artery-banded mice and in a rat model of genetic congestive heart failure (Zhang *et al.* 1998), as well as in failing human hearts (Owen *et al.* 2001, Mittmann *et al.* 2002). *In vitro*, RGS4 overexpression inhibits phenylephrine and endothelin-1-mediated induction of atrial natriuretic factor and myosin light chain-2 genes (Tamirisa *et al.* 1999) and attenuates sphingosine 1-phosphate receptor signaling (Cho *et al.* 2003). *In vivo*, RGS4 overexpression in ventricular tissue impairs the ability of the heart to adapt to acute ventricular pressure overload and cardiac hypertrophy caused by transverse aortic constriction (Rogers *et al.* 1999). Cardiac RGS4 is involved in diabetic cardiomyopathy since its overexpression confers resistance to streptozotocin-induced cardiac fetal gene induction (Harris *et al.* 2004). In the nervous system, RGS4 is also highly expressed and regulated by physiological and pharmacological agents (Ingi & Aoki 2002, Neubig & Siderovski 2002, Larminie *et al.* 2004). RGS4 mRNA levels are consistently decreased in the prefrontal cortex of schizophrenic patients (Mirnics *et al.* 2001). Linkage analysis studies indicated that several nucleotide polymorphisms in the RGS4 promoter region may be associated with schizophrenia (Chowdari *et al.* 2002, Morris *et al.* 2004), although more studies are required to confirm these associations (O'Donovan *et al.* 2003).

We hypothesize that genes preferentially/exclusively expressed in the rat adrenal zona glomerulosa may be involved in specific responses to proliferation and steroidogenesis of this adrenal gland zone. We used differential display to screen for these genes and observed that RGS4 was preferentially expressed in the rat adrenal zona glomerulosa. Since no studies have been reported regarding RGS4 expression and role in the adrenal cortex, we have conducted further studies to analyze RGS4 expression, regulation, and function in adrenocortical cells. We performed detailed analysis of RGS4 expression in rat adrenal gland and analyzed its regulation by salt diet intake manipulation and Ang II infusion *in vivo*. We also studied RGS4 regulation, the intracellular signaling mechanisms involved, and its role in aldosterone secretion using the H295R human adrenal cell line as our *in vitro* experimental model.

Materials and Methods

Materials

Ang II was obtained from Sigma Chemical Co. Ionomycin, nifedipine, SKF-96365 (SKF), calmidazolium, KN-93, autocamtide-2 cell permeable inhibitory peptide, phorbol-12-myristate-13-acetate (PMA), bisindolylmaleimide I (GF 109203X), calphostin C, chelerythrine, Gö 6976, Gö 6983, Ro-31-8220, Ro-32-0432, and myristoylated protein kinase C (PKC) inhibitor peptide (20–28) were obtained from EMD Biosciences (San Diego, CA, USA). W-7 and STO-609 were obtained from Tocris (Ellisville, MO, USA). Merck Laboratories generously provided Losartan. Human adrenal total RNA was obtained from several sources: hAd1 (59-year-old male donor) from BioChain Institute Inc. (Hayward, CA, USA), hAd2 (pooled from 61 male/female Caucasian donors, ages 15–61) from BD Biosciences (Mountain View, CA, USA), and hAd3 (30-year-old female donor) from Stratagene (La Jolla, CA, USA).

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee of the G V Montgomery VA Medical Center and by the Central Office of the Department of Veterans Affairs. Animal use and husbandry were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and conducted in AAALAC-accredited facilities.

Male Sprague–Dawley rats (3 months old) were obtained from Harlan Sprague–Dawley (Indianapolis, IN, USA) and maintained on standard rat chow (Teklad, Harlan, Indianapolis, IN, USA) and tap water in an environment with 12 h light:12 h darkness cycles.

Salt diet manipulation Rats ($n=6$ per group) were fed *ad libitum* a standard normal salt diet (0.3% NaCl; Teklad, Harlan, Madison, WI, USA), low-salt diet (0.03% NaCl), or high-salt diet (standard chow plus 0.9% saline to drink) for 2 or 15 days.

Ang II infusion Rats ($n=6$ per group) were anesthetized by isoflurane gas and a catheter was placed in the femoral vein. The catheter was exteriorized at the back of the neck and rats were allowed to recover for 2 days. Conscious unrestrained rats were connected to an infusion pump and infused for 2 or 6 h with Ang II in saline at a dose of 100 ng/kg per min at rate of 500 μ l/h. Control rats received saline.

At the end of the experimental protocols, rats were anesthetized with isoflurane, adrenal glands removed, excised of fat, flash frozen in liquid nitrogen, and stored at -80°C .

Northern blot

Northern blots were performed as previously described (Zhou *et al.* 1995) with the following modifications. Nine

micrograms of total RNA from capsule and core fractions of rat adrenal gland were separated by formaldehyde/formamide denaturing electrophoresis and transferred to Zeta Probe GT nylon membrane (Bio-Rad). PCR products were labeled using the Strip-EZ DNA kit (Ambion, Austin, TX, USA). Membranes were exposed to autoradiography film. Films were scanned and quantified with a Kodak Image Station 440 using the 1D Kodak image analysis software. Membranes were stripped and reprobed with GAPDH as loading control.

In situ hybridization

In situ hybridizations were performed as we previously described (Wotus *et al.* 1998, Daido *et al.* 2003) using the Sure-Site kit (EMD Biosciences) with the following modifications. Sections were washed once with $2\times$ SSC for 30 min at 55 °C followed by one wash in $1\times$ SSC plus one more in $0.5\times$ SSC under similar conditions. Sections were air-dried and exposed to autoradiography film. Films were scanned with a Kodak Image Station 440.

Cell culture

H295R human adrenocortical cells (Bird *et al.* 1993) were cultured in H295R complete medium containing Dulbecco's modified Eagle's medium (DMEM):F12 (1:1) supplemented with 2% Ultrosor G (Biosepra, Villeneuve-la-Garenne, France), Insulin/Transferrin/Selenium-Plus (Discovery Labware, Bedford, MA, USA), and antibiotic/antimycotic mixture (Invitrogen) as we previously described (Romero *et al.* 2004), until subconfluent in 6-well plates. Medium was replaced with 3 ml fresh medium containing various agents and cultured for 3 h more unless otherwise indicated. At the end of the incubation period, medium was removed and saved for steroid determination. To study the intracellular signaling pathways involved in RGS4 regulation, we used several inhibitors of the calcium/calmodulin/calmodulin-dependent protein kinase (CaMK) and PKC pathways (detailed in the Results section) which were added 30 min before Ang II addition. Reference sources used to select the inhibitor concentration used are indicated in Supplementary material Table 1 (see supplementary data in the online version of

the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol 194/issue 2>); preference was given to previous reports in the following order: (1) H295R cells, (2) other adrenocortical cell systems, (3) other cell systems.

RNA extraction and RT-PCR

Adrenal gland total RNA was extracted with Tri-Reagent (MRC, Cincinnati, OH, USA), resuspended in diethyl pyrocarbonate-treated H_2O , DNase treated with Turbo DNA-free kit (Ambion). H295R cell total RNA was extracted with the RNeasy Micro kit (Qiagen) and on-column DNase 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. Primers were designed with Primer3 software (Rozen & Skaletsky 2000) and are shown in Table 1. Real-time PCR was performed with 1 µl RT product, 1 µl titanium Taq DNA polymerase (Clontech), 1:20 000 dilution SYBR Green I (Molecular Probes, Carlsbad, CA, USA), 0.2 mM dNTPs, and 0.1 µM of each primer. Cycling conditions were 1 min at 95 °C, 50 cycles of 15 s at 95 °C, 15 s 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 (Pfaffl 2001) 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. The specificity of the PCR products was confirmed by melting temperature determination of the PCR product, and restriction enzyme digestion followed by high-resolution electrophoretic analysis in 4% NuSieve 3:1 agarose gels (Cambrex, Rockland, ME, USA) of PCR products (Supplementary Fig. 1; see supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol 194/issue 2>)).

Plasmids

pGEM-T (Promega) plasmid carrying a fragment of rat RGS4 mRNA (bases 1397–2919 from NM_017214) was

Table 1 Real-time PCR primers

	Accession number	Primers	Product size (bp)
Gene			
Rat <i>RGS4</i>	NM_017214	Forward 5'-CAGGCAACAAAAGAGGTGGAAC-3' Reverse 5'-TCTTCTGGGCTTCATCAAAAC-3'	100
Rat <i>GAPDH</i>	NM_017008	Forward 5'-AAGATGGTGAAGGTCCGTGT-3' Reverse 5'-GTTGATGGCAACAATGTCCACT-3'	99
Human <i>RGS4</i>	NM_005613	Forward 5'-CTGCAAAAATCTGATTCCTGTG-3' Reverse 5'-CCCATTCTTGACTTCCTCTTG-3'	100
Human <i>GAPDH</i>	NM_002046	Forward 5'-CCCCTTCATTGACCTCACTAC-3' Reverse 5'-GATGACAAGCTTCCCGTTCTC-3'	103

used for probe generation for northern blot or *in situ* hybridization. A plasmid containing the human RGS4 insert in pDNR-1r was obtained from the University of Missouri at Rolla cDNA Resource Center (www.cdna.org). RGS4 was transferred from pDNR-1r to the retroviral BD Creator acceptor vector pLP-LNCX using the manufacturer's suggested protocol. The pVSV-G plasmid was purchased from Clontech.

Retroviral production and infection

VSV-G-pseudotyped retroviral particles were generated by transient transfection in GP2-293 cells as previously described (Romero *et al.* 2006). GP2-293 cells (Clontech) were cultured in DMEM supplemented with 10% fetal bovine serum until 60–70% confluent, then transfected with retroviral vector and pVSV-G plasmids (0.25 µg/cm², 1:1 molar ratio) using Trans-IT 293 transfection reagent (2 µl/µg DNA, Mirus Bio, Madison, WI, USA). Medium was replaced after 4 h and cells 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 polyether sulfone (PES) membrane, aliquoted, and stored at –80 °C. For retroviral infection, confluent H295R cells were split 1:3 in 6-well plates and cultured overnight. Retroviral supernatant was diluted 1:1 with fresh medium, added to cells, and cultured for 24 h. Medium was 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 weeks in the absence of selecting agent before performing the experiments to avoid any confounding effect due to the selecting antibiotic.

Aldosterone ELISA

Aldosterone was measured in cell culture supernatants by ELISA using an MAB developed in our laboratory as previously described (Gomez-Sanchez *et al.* 1987). Assay sensitivity was 20 pg/ml.

Statistical analysis

All results were expressed as mean ± S.E.M. Two groups were compared by *t*-test and multiple groups were analyzed by one-way ANOVA followed by Tukey's *post hoc* comparisons or two-way ANOVA followed by Bonferroni 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 tested by *F*-test; values were tested by two-way ANOVA followed by Bonferroni comparisons. All experiments were repeated at least twice in triplicates. 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, USA).

Results

RGS4 is expressed in the rat adrenal zona glomerulosa

To confirm our initial observation that RGS4 was preferentially expressed in the rat adrenal zona glomerulosa, we performed northern blot and *in situ* studies in the rat adrenal gland. We performed a northern blot using the capsule (outer cortex comprising zona glomerulosa and some zona fasciculata) and core (internal cortex and medulla) of the rat adrenal gland. RGS4 was preferentially expressed in the capsule fraction with negligible amounts observed in the core fraction (Fig. 1A). Membranes were stripped and reprobed with a GAPDH probe as an internal loading control to ensure that similar RNA amounts were loaded in each lane.

To more precisely localize RGS4 expression in the rat adrenal gland, we performed an *in situ* hybridization with a homologous rat RGS4 probe. RGS4 mRNA expression was localized in the zona glomerulosa of rat adrenal gland (Fig. 1B). The negative control sense probe showed no signal (Fig. 1B), indicating the specificity of the antisense RGS4 probe.

RGS4 is regulated by salt intake in rat adrenal

One of the main physiological modulators of adrenal gland zona glomerulosa physiology is salt intake. To study whether salt intake regulates RGS4 expression in adrenal gland, rats were placed on low- or high-salt diet for 2 or 15 days and RGS4 mRNA quantified in adrenal glands by real-time RT-PCR. Low-salt diet caused a continuous increase in adrenal gland

Figure 1 RGS4 expression in rat adrenal gland. (A) Rat adrenal glands were separated into capsules and core for RGS4 and GAPDH mRNA expression analysis by northern blot. (B) Rat adrenal gland sections were probed with a RGS4 antisense probe or a sense control probe by *in situ* hybridization. Representative figures from experiments repeated at least six times.

RGS4 mRNA expression reaching a 66% increase after 15 days of treatment (1.01 ± 0.08 vs 1.66 ± 0.17 , $P < 0.05$; Fig. 2A). In contrast, high-salt diet did not modify RGS4 mRNA levels up to 15 days of treatment.

RGS4 is regulated by Ang II infusion in rat adrenal

To study whether Ang II, a well-known aldosterone secretagogue and modulator of adrenal zona glomerulosa physiology, regulates RGS4 expression in adrenal gland, rats were infused with Ang II. Conscious chronically catheterized rats were infused with Ang II for 2 or 6 h, and adrenal RGS4 mRNA was quantified by real-time RT-PCR. Ang II infusion caused an increase in RGS4 mRNA expression reaching a 131% increase after 6 h of treatment (1.00 ± 0.10 vs 2.31 ± 0.34 , $P < 0.05$; Fig. 2B).

RGS4 is upregulated by Ang II in H295R cells

To further study the molecular mechanisms involved in the regulation and role of RGS4 in aldosterone secretion by adrenal

Figure 2 RGS4 mRNA expression regulation by sodium diet (A) or angiotensin II infusion (B). Rats were placed on low or high sodium diets for 2 or 15 days (A) or infused with 100 ng/kg per min angiotensin II for 2 or 6 h (B). Adrenal glands were removed and RGS4 mRNA quantified by real-time RT-PCR. * $P < 0.05$ versus rats in control diet (A) or versus rats infused with saline (B), $n = 6$ per group.

cells, H295R human adrenocortical cells were used as the experimental *in vitro* model. It is the only adrenal cell line that expresses all of the steroidogenic enzymes required for the synthesis of aldosterone from cholesterol and has a steroid secretion pattern and regulation similar to that of primary adrenal cell cultures (Railey *et al.* 1994, 2004). H295R adrenocortical cells, as well as normal human adrenal glands, expressed RGS4 mRNA as detected by agarose gel electrophoresis of PCR products (Fig. 3A). Controls with no reverse transcriptase showed no amplification. GAPDH was used as a housekeeping control gene.

To determine whether RGS4 mRNA levels are subjected to regulation by Ang II in H295R cells as in rat adrenal glands, H295R human adrenocortical cells were incubated with 100 nM Ang II for increasing periods up to 72 h and RGS4 mRNA expression was quantified by real-time PCR (Fig. 3B). Ang II caused a 25-fold increase in RGS4 mRNA levels 3 h after hormone stimulation. RGS4 mRNA then declined to basal levels within 24 h and remained suppressed for up to 72 h after the initiation of Ang II treatment. When H295R cells were treated with Ang II for shorter periods, it was observed that RGS4 mRNA levels reached maximal stimulation after 4-h treatment (Fig. 3C). To study the effect of different doses of Ang II on RGS4 mRNA expression, H295R cells were incubated with increasing concentrations of Ang II for 3 h and RGS4 mRNA quantified (Fig. 3D). Ang II (0.1–1000 nM) dose dependently increased RGS4 mRNA levels. To confirm the specificity of Ang II treatment on RGS4 mRNA regulation, H295R cells were treated with the Ang II receptor type 1 (AT1-R) blocker losartan (10 μ M) in the presence or absence of Ang II (10 nM) for 30 min (Fig. 3E). Losartan completely abolished (98% inhibition) Ang II-mediated RGS4 mRNA upregulation, indicating that AT1-R specifically mediates RGS4 mRNA regulation by Ang II. The CaMK and PKC pathways are primary mediators of Ang II intracellular signaling in adrenal cells (Barrett *et al.* 1989, Ganguly & Davis 1994, Bassett *et al.* 2004, Spat & Hunyady 2004). To determine whether either of these pathways is involved in the regulation of RGS4 mRNA expression in H295R cells, we performed the following studies.

Calcium signaling is involved in Ang II-mediated RGS4 upregulation in H295R cells

To analyze whether calcium channel blocking would alter Ang II-mediated RGS4 upregulation, H295R cells were preincubated with the L-type Ca^{2+} channel selective blocker nifedipine before Ang II stimulation (Fig. 4A). Nifedipine caused a 55% decrease in Ang II-mediated RGS4 upregulation (100.0 ± 8.9 vs 45.1 ± 2.0 , $P < 0.05$).

To verify the involvement of calcium signaling on RGS4 mRNA upregulation, H295R cells were preincubated with SKF, an inhibitor of both receptor-mediated and voltage-gated calcium influx entry (Merritt *et al.* 1990), before Ang II stimulation. SKF caused a 36% decrease in Ang II-mediated

Figure 3 RGS4 expression and regulation in H295R cells. (A) RGS4 mRNA expression in H295R cells and human adrenal glands detected by agarose gel electrophoresis of RT-PCRs in the presence (+) or absence (–) of reverse transcriptase. GAPDH mRNA expression was used as control. hAd1: 59-year-old male donor, hAd2: pooled from 61 male/female white donors, ages 15–61, hAd3: 30-year-old female donor. (B and C) H295R cells were incubated with or without 100 nM angiotensin II for 0–72 h (B) or 0–6 h (C) periods, RNA extracted, and then quantified by real-time RT-PCR. RGS4 mRNA was normalized by GAPDH mRNA expression and expressed as fold increase versus time=0. * $P<0.05$ versus basal, $n=3$. (D) H295R cells were incubated with increasing concentrations of Ang II for 3 h, RNA extracted, and then quantified by real-time RT-PCR. RGS4 mRNA was normalized by GAPDH mRNA expression and expressed as fold increase versus time=0. * $P<0.05$ versus control, $n=3$. (E) H295R cells were treated with 10 μ M losartan or vehicle (control) for 30 min and then incubated in the presence or absence of angiotensin II (Ang II, 10 nM) for 3 h, RNA extracted, and then quantified by real-time RT-PCR. RGS4 mRNA was normalized by GAPDH mRNA expression and expressed as percentage of angiotensin II stimulation. * $P<0.05$ versus basal–control, # $P<0.05$ versus Ang II–control, $n=3$.

RGS4 mRNA upregulation (100.0 ± 7.6 vs 63.4 ± 6.4 , $P<0.05$; Fig. 4B).

Furthermore, when H295R cells were incubated with the calcium ionophore ionomycin (1 μ M) for 3 h, RGS4 mRNA levels were increased 2.1-fold when compared with control cells (1.00 ± 0.12 vs 2.17 ± 0.29 , $P<0.05$).

Calmodulin and CaMKK/CaMK mediates RGS4 mRNA upregulation by Ang II in H295R cells

Calcium mediates most of its biological effects through binding to the calcium-binding protein, calmodulin, causing a conformational change allowing calmodulin to interact and

Figure 4 Effect of Ca^{2+} channel blockers on RGS4 mRNA 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 angiotensin II (Ang II, 100 nM) for 3 h. RNA was extracted and then quantified by real-time RT-PCR. RGS4 mRNA was normalized by GAPDH mRNA expression and expressed as fold increase or percentage of angiotensin II stimulation. * $P < 0.05$ versus basal-control, # $P < 0.05$ versus control for its treatment, $n = 3$.

modify the activity of its target proteins. We studied the effect of two calmodulin antagonists, W-7, and calmidazolium, on basal and stimulated conditions. W-7 and calmidazolium caused a 66% (100.0 ± 4.4 vs 44.1 ± 1.5 , $P < 0.05$) and 67% (100.0 ± 4.4 vs 32.8 ± 1.36 , $P < 0.05$) decrease respectively in Ang II-mediated RGS4 mRNA induction (Fig. 5A).

Once complexed with calcium, calmodulin can interact with calmodulin kinases (CaMK), allowing phosphorylation of the inhibitory subunit by CaMK regulatory subunit and causing activation of the protein kinase. CaMK kinase (CaMKK) is an upstream regulator of CaMK that is also

Figure 5 Effect of calmodulin antagonists and CaMK and CaMK inhibitors on RGS4 expression. H295R cells were treated with 50 μM W-7 (A), 10 μM calmidazolium (A), 1 $\mu\text{g/ml}$ STO-609 (B), 5 μM KN-93 (B), 10 μM autocamtide-2 (B), or vehicle for 30 min and then incubated in the presence or absence of angiotensin II (Ang II, 100 nM) for 3 h. RNA was extracted and then quantified by real-time RT-PCR. RGS4 mRNA was normalized by GAPDH mRNA expression and expressed as percentage of Angiotensin II stimulation. * $P < 0.05$ versus basal-control, # $P < 0.05$ versus control for its treatment, $n = 3$.

regulated by calmodulin. CaMKs are expressed in the adrenal gland and are among the main effectors of calcium intracellular signaling in this gland (Barrett *et al.* 1989, Ganguly & Davis 1994, Condon *et al.* 2002). Pretreatment of H295R cells with the CaMKK-specific inhibitor STO-609 caused a 36% decrease in Ang II-mediated RGS4 mRNA induction (100.0 ± 5.1 vs 64.0 ± 2.6 , $P < 0.05$; Fig. 5B).

To analyze whether CaMK mediates RGS4 mRNA induction, H295R cells were pretreated with the CaMK-specific inhibitors KN-93 or autocamtide-2. Both KN-93 and autocamtide-2 caused a 42% (100.0 ± 5.1 vs 58.5 ± 2.6 , $P < 0.05$) and 38% (100.0 ± 5.1 vs 62.4 ± 2.0 , $P < 0.05$) decrease respectively in Ang II-mediated RGS4 mRNA induction (Fig. 5B).

PKC mediates Ang II-mediated RGS4 mRNA upregulation in H295R cells

To study the role of PKC in RGS4 mRNA upregulation, we performed experiments involving activation and inhibition of

this protein kinase. Incubation of H295R cells with increasing concentrations of the PKC activator PMA caused a dose-dependent increase in RGS4 mRNA levels, reaching maximum levels at 100 nM PMA (23.65 ± 0.63 vs 1.00 ± 0.05 , $P < 0.05$; Fig. 6A). Since exogenous activation of PKC increases RGS4 mRNA levels, we studied whether PKC inhibitors could modify Ang II-mediated RGS4 mRNA upregulation. The generic PKC inhibitors bisindolylmaleimide I and Gö 6983 almost completely blocked Ang II-mediated RGS4 mRNA upregulation (100.0 ± 3.3 vs 16.4 ± 0.7 , $P < 0.05$, vs 8.71 ± 0.3 ,

$P < 0.05$ respectively; Fig. 6B). Other PKC inhibitors, calphostin C, chelerythrine, Gö 6976, Ro-31-8220, Ro-32-0432, and the PKC inhibitory peptide (20–28) all decreased Ang II-mediated RGS4 mRNA upregulation (Fig. 6B), suggesting a critical role of PKC in RGS4 mRNA level modulation.

RGS4 overexpression decreased aldosterone secretion in H295R cells

To examine the effect of RGS4 upregulation on aldosterone secretion, we used retroviruses expressing RGS4 under control of the cytomegalovirus (CMV) promoter to generate H295R cells that stably overexpress RGS4 (H295R–RGS4 cells). H295R–RGS4 significantly overexpressed RGS4 mRNA ($P < 0.05$, Fig. 7 insert). To study whether RGS4 overexpression alters aldosterone secretion in response to Ang II, H295R and H295R–RGS4 cells were incubated with increasing concentrations of Ang II, and the cell culture supernatants were assayed for aldosterone. RGS4 overexpression caused a significant decrease in aldosterone secretion at all doses of Ang II tested (Fig. 7). RGS4 overexpression reduced basal (22.3 ± 4.6 vs 0.38 ± 1.44 , $P < 0.05$) and maximal stimulation (108.4 ± 6.7 vs 50.7 ± 4.3 , $P < 0.05$) aldosterone secretion.

Discussion

In the present study, we demonstrate that: (1) RGS4 is expressed in the rat adrenal gland exclusively in the zona glomerulosa, (2) rat adrenal RGS4 mRNA is upregulated *in vivo* by low-salt diet intake and Ang II infusion, (3) RGS4 is expressed in H295R human adrenocortical cells and is

Figure 6 Effect of protein kinase C on RGS4 mRNA expression. H295R cells were incubated with increasing concentrations of PKC activator PMA for 3 h (A) or treated with different PKC inhibitors or vehicle for 30 min and then incubated in the presence or absence of angiotensin II (Ang II, 100 nM) for 3 h (B). RNA was extracted and then quantified by real-time RT-PCR. RGS4 mRNA was normalized by GAPDH mRNA expression and expressed as percentage of angiotensin II stimulation. * $P < 0.05$ versus control, # $P < 0.05$ versus Ang II, $n = 3$.

Figure 7 Effect of RGS4 overexpression on aldosterone secretion. H295R cells were infected with retroviruses expressing RGS4, selected, and incubated with increasing concentrations of Angiotensin II for 24 h. Aldosterone concentration was measured in medium supernatant and cells lysed to quantify total protein. Insert shows RGS4 mRNA overexpression in H295R–RGS4 cells. * $P < 0.05$ versus control cells, $n = 3$.

upregulated by Ang II, (4) Ang II-mediated RGS4 mRNA upregulation in H295R cells is mediated by the calcium/calmodulin/CaMK and PKC pathways, and (5) RGS4 overexpression in H295R cells caused a decrease in Ang II-mediated aldosterone secretion.

Hormonal signals are crucial for the functional 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 as important as their initiation to achieve homeostasis at the 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 upon exchange of GDP by GTP are activated and available to interact with target effector proteins to transduce the Ang II signal from the extracellular space to the intracellular compartment. Our results indicate that *RGS4* is a gene in the adrenal gland that is exclusively expressed in the zona glomerulosa and whose expression is regulated *in vivo* by physiological modulators of zona glomerulosa cells, salt intake, and Ang II. RGS4 proved to be regulated by Ang II in H295R cells *in vitro* and its overexpression caused a decrease in Ang II-mediated aldosterone secretion. Since the main physiological function of RGS proteins is to increase the rate of G α protein inactivation and consequently the downregulation of the intracellular signal triggered by the GPCR, RGS4 is probably crucial for the physiological termination of Ang II signal in adrenal cells, prevention of an exaggerated or prolonged Ang II signal, and promotion of homeostasis.

We have recently reported that Ang II upregulates RGS2 expression in H295R human adrenocortical cells and exerts similar physiological effects as RGS4 on adrenal steroidogenesis regulation (Romero *et al.* 2006). The regulation of RGS2 and RGS4 mRNA levels and the intracellular signaling pathways involved, although similar, are unique. Ang II caused a continuous increase in RGS4 mRNA levels up to 4 h after treatment, while RGS2 mRNA reached maximal stimulation levels within 1 h (Romero *et al.* 2006). These results agree with the few reports in which time-response curves of RGS2 and RGS4 mRNA levels were generated. Taymans *et al.* (2004) reported that dopamine receptor 2 blockade caused regulation of RGS2 and RGS4 mRNA levels in rat brain in opposite directions, with RGS4 mRNA levels upregulated more slowly than RGS2 levels. Although Ang II-mediated RGS4 mRNA upregulation in adrenal cells lags that of RGS2, the degree of stimulation is much higher reaching 25-fold when compared with only 5-fold for RGS2 (Romero *et al.* 2006). Both Ang II-mediated RGS4 and RGS2 mRNA upregulation are mediated by the calcium/calmodulin/CaMKK/CaMK intracellular signaling pathway. However, PKC seems to be a key mediator in Ang II-mediated RGS4 mRNA upregulation, while this protein

kinase does not seem to play a significant role in the regulation of RGS2 mRNA in H295R cells (Romero *et al.* 2006).

To study the role of RGS4 in Ang II-mediated aldosterone secretion, we overexpressed RGS4 in H295R cells. RGS4 overexpression caused a decrease in Ang II-mediated aldosterone production as well as in basal aldosterone secretion in the absence of added Ang II. This finding is not surprising since H295R cells, like adrenal zona glomerulosa cells *in vivo*, have an endogenous renin-angiotensin system that generates Ang II under basal conditions promoting basal aldosterone secretion in the absence of exogenous stimuli (Hilbers *et al.* 1999). RGS4 overexpression in H295R cells reduced maximal Ang II-mediated aldosterone secretion but did not modify the responsiveness of the system, since it did not modify Ang II pEC₅₀ for aldosterone secretion. The effects of RGS4 overexpression on adrenal cell steroidogenesis are similar to those we previously reported for RGS2 (Romero *et al.* 2006). Although there was a significant increase in RGS4 mRNA levels in H295R-RGS4 overexpressing cells, the fact that RGS4 did not blunt Ang II-mediated steroidogenesis may indicate that this RGS protein may be necessary but not solely sufficient to turn off Ang II signaling in adrenal cells. Other cellular processes, such as Ang II signaling and Ang II receptor desensitization and downregulation, and Ang II receptor internalization are known to occur in H295R cells as well as adrenocortical cells in response to Ang II (Bianchi *et al.* 1986, Penhoat *et al.* 1988, Naville *et al.* 1993, Bird *et al.* 1994, Boulay *et al.* 1994, Ouali *et al.* 1997, Richard *et al.* 1997, Spat & Hunyady 2004) and complement RGS4 role in the regaining of cellular homeostasis after Ang II stimulation.

Ang II upregulates RGS4 mRNA levels through the calcium/calmodulin/CaMKK/CaMK and PKC pathways. It has been reported that Ang II upregulates CaMK activity in bovine zona glomerulosa cells (Fern *et al.* 1995, Gambaryan *et al.* 2006). CaMK inhibitors inhibit Ang II-mediated aldosterone secretion (Denner *et al.* 1996, Gambaryan *et al.* 2006) and aldosterone synthase expression (Pezzi *et al.* 1997, Condon *et al.* 2002). More recently, it has been shown by cotransfection reporter assays that CaMKI may be the CaMK isoform involved in Ang II-mediated aldosterone synthase expression regulation (Condon *et al.* 2002). PKC negatively affects Ang II-mediated aldosterone secretion and aldosterone synthase expression (Bird *et al.* 1998, LeHoux & Lefebvre 1998, Hajnoczky *et al.* 1992, LeHoux *et al.* 2001). PKC activators decrease and PKC inhibitors increase aldosterone synthase expression (Bird *et al.* 1998, LeHoux & Lefebvre 1998, LeHoux *et al.* 2001). More recently, PKC ϵ has been proposed to be the PKC isozyme involved in the regulation of aldosterone synthase in H295R cells (LeHoux & Lefebvre 2006). RGS4 has a complex interaction with the intracellular signaling pathways that regulate its expression. While the calmodulin/CaMKK/CaMK signaling pathway is a positive modulator of aldosterone secretion and aldosterone synthase expression, it also upregulates RGS4 expression which then functions as a negative feedback of Ang II-mediated

aldosterone secretion. On the other hand, the decrease in aldosterone secretion and aldosterone synthase expression produced by PKC may be mediated by its regulation of RGS4 expression. Perhaps, PKC activation decreases aldosterone secretion by upregulating RGS4 expression, thus terminating Ang II-mediated signaling that increases aldosterone synthesis.

A limitation of the present report is that RGS4 regulation was detected at the mRNA levels instead of the protein level. Endogenous RGS4 levels are very low and below the detection limit of currently available antibodies, as we have observed in rat adrenal gland and H295R cells, and as other researchers have reported in several other cell lines (Ingi *et al.* 1998). Plasmid-driven expression of RGS4 protein in mammalian cells have a half-life of only 45 min (Davydov & Varshavsky 2000) due to its rapid degradation by the ubiquitin-dependent N-end rule pathway (Davydov & Varshavsky 2000, Lee *et al.* 2005). The short half-life of RGS4 protein makes it very likely that RGS4 protein levels reflect those of its mRNA, and the determination of RGS4 mRNA levels are an achievable and valid extrapolation for the study of the regulation of RGS4 levels in adrenal cells.

In the present report, we show that RGS4 is zona glomerulosa-specific gene of adrenal gland, is upregulated by low sodium intake and Ang II, and is involved in adrenal cell aldosterone secretion regulation. These findings and our previous ones regarding RGS2 elucidate a new mechanism by which Ang II intracellular signaling is terminated and the Ang II receptor desensitized independently of Ang II receptor downregulation by internalization within the adrenal cell. We speculate that RGS2 and RGS4 are crucial for the regulation of aldosterone synthesis and that alteration in either their expression or activity due to loss or gain of function mutations may lead to abnormal adrenal GPCR signaling and aldosterone synthesis.

Aldosterone exerts its effect not only in epithelial target tissues, where its main function is regulation of sodium/potassium and water balance, but also in non-epithelial tissues such as the brain and the heart. High aldosterone levels have been associated with severe target organ injury in the heart and the kidney of hypertensive patients with primary aldosteronism (Rossi *et al.* 1997, 2006, Tanabe *et al.* 1997). In addition, excess aldosterone in non-hypertensive patients has been reported to correlate with an increased risk to suffer an increase in blood pressure or develop hypertension (Vasan *et al.* 2004, Newton-Cheh *et al.* 2007). Alterations in Ang II-mediated aldosterone synthesis and secretion by the adrenal gland due to RGS4 alterations in its expression levels and/or activity may underlie or exacerbate deleterious effects of aldosterone caused by its excessive synthesis and secretion.

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