

## Specific Regulation of RGS2 Messenger RNA by Angiotensin II in Cultured Vascular Smooth Muscle Cells

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### ABSTRACT

The effects of angiotensin II (Ang II) are mediated primarily by Ang II type 1 receptors, which in turn are coupled to heterotrimeric G proteins. After receptor activation, the  $G_\alpha$  and  $G_{\beta\gamma}$  subunits dissociate, contributing to the signaling cascades involving protein kinase C (PKC) activation. Regulators of G protein signaling (RGS proteins) comprise a class of proteins that have been shown to negatively regulate the  $G_\alpha$  subunit. We examined which RGS sequences were expressed in vascular smooth muscle cells and which of these were regulated by Ang II. Reverse transcription-polymerase chain reaction showed that of 16 RGS sequences screened, six RGS transcripts (RGS2, 3, 10, 11, and 12 and GAIP) were present. Northern blot analysis demonstrated that RGS3, 10, and 12 and GAIP were not regulated by Ang II at the mRNA level. In contrast, RGS2 mRNA was rapidly and dose dependently increased ( $395 \pm 24\%$  peak, 45 min) by Ang II but returned to baseline level by 6

to 8 h. Phorbol-12-myristate-13-acetate, a PKC activator, robustly increased RGS2. This signal was attenuated by the PKC inhibitor GF 109203X ( $50 \pm 4\%$ ) and by phorbol-12,13-dibutyrate-mediated down-regulation of PKC ( $48 \pm 13\%$ ). Tyrosine kinase inhibition and calcium deprivation did not affect the up-regulation of RGS2 mRNA after Ang II stimulation. Actinomycin D treatment inhibited both Ang II- and phorbol-12-myristate-13-acetate-stimulated RGS2 up-regulation, suggesting activation of transcription by these agonists. The stability of RGS2 mRNA did not appear to be affected by Ang II. Thus, RGS2 is a likely candidate for negative regulation of the G proteins coupled to the Ang II type 1 receptor in vascular smooth muscle cells. Regulation of this protein may be of critical importance in modulating the role of Ang II in vascular disease.

Angiotensin II (Ang II) activates an impressive array of signaling pathways in vascular smooth muscle cells (VSMCs), predominantly through the seven-transmembrane, heterotrimeric G protein-coupled angiotensin II type 1 receptor ( $AT_{1A}R$ ). Binding of Ang II to the  $AT_{1A}R$  causes a biphasic response with a rapid but transient activation of phosphatidylinositol-specific phospholipase C (PLC), producing inositol trisphosphate ( $IP_3$ ) and diacylglycerol followed by a prolonged activation of phospholipase D (PLD) (Griendling et al., 1997). Production of  $IP_3$  occurs within seconds and reaches maximal activation at 15 s, after which it returns to baseline levels. This coupling of the  $AT_{1A}R$  to PLC is mediated through  $G_{\alpha_{q/11\beta\gamma}}$  and  $G_{\alpha_{12\beta\gamma}}$  (Ushio-Fukai et al., 1998), but

the mechanisms responsible for termination of the signal remain largely unknown.

Regulators of G protein signaling (RGS proteins) comprise a novel class of proteins that act as GTPase-activating proteins (GAPs) for heterotrimeric G protein  $\alpha$ -subunits in vitro. Approximately 20 mammalian RGS sequences have been identified, and all contain a highly conserved carboxyl-terminal domain of  $\sim 125$  amino acids (RGS domain). It is this domain that is integral to the catalytic activity of the RGS proteins (Popov et al., 1997), whereas the highly divergent sequences flanking the RGS domain may determine their specificity. Several recent studies have shown that apart from the negative regulation of  $G_\alpha$  subunits, the RGS proteins may also interact with  $\beta\gamma$ -subunits (Bunemann and Hosey, 1998; Snow et al., 1998). Most RGS proteins are present in multiple tissues, and most tissues and cells have multiple RGS proteins. Moreover, most RGS proteins act as GAPs on more than one  $G_\alpha$  subunit. The reason for multiple RGS proteins within a cell type is unknown but may be

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**ABBREVIATIONS:** Ang, angiotensin; VSMC, vascular smooth muscle cell;  $AT_{1A}R$ , angiotensin II type 1 receptor; PLC, phospholipase C;  $IP_3$ , inositol trisphosphate; PLD, phospholipase D; GAP, GTPase-activating protein; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; RT, reverse transcription; PCR, polymerase chain reaction; RGS, regulators of G protein signaling.

related to specificity of coupling. Recently, Xu et al. (1999) described variability in RGS4-mediated inhibition of calcium release stimulated by different  $G_q$ -coupled receptors, suggesting that RGS proteins exhibit receptor selectivity.

The existence and identity of putative RGS proteins that interact with  $AT_{1A}$ Rs have not been demonstrated in any cell type. However, the transient nature of PLC activation, together with the fact that  $AT_{1A}$ Rs couple to  $G_{\alpha q}$ , which is a substrate for RGS2 (Heximer et al., 1997), RGS4 (Heximer et al., 1997), and RGS3 (Neill et al., 1997), suggests that  $AT_{1A}$ R function is likely to be regulated by an RGS protein. The available tools with which to study this interaction are limited. In previous work, we have shown that many proteins involved in  $AT_{1A}$ R signaling cascades are also regulated at the mRNA level by a 4- to 16-h incubation with Ang II. Thus, prolonged Ang II stimulation down-regulates  $AT_{1A}$ R (Lassègue et al., 1995) and  $G_{\alpha q/11}$  mRNA levels (Kai et al., 1996) and up-regulates G protein-coupled receptor kinase 5 mRNA (Ishizaka et al., 1997).

We hypothesized that RGS proteins are involved in Ang II signaling in VSMCs and that Ang II would regulate at least one RGS at the mRNA level. Here we report that rat VSMCs express six RGS sequences: RGS2, 3, 10, 11, and 12 and GAIP. Of these proteins, only RGS2 mRNA is significantly

regulated within 24 h of Ang II stimulation (100 nM). Ang II-induced RGS2 up-regulation is partially protein kinase C (PKC)-dependent, tyrosine kinase- and calcium-independent, and transcriptionally regulated. The unique and rapid regulation of RGS2 by Ang II suggests an important role for RGS2 in the  $AT_{1A}$ R signaling cascade in VSMCs.

## Experimental Procedures

**Materials.** Losartan was a gift from Dr. R. D. Smith (DuPont Merck Pharmaceutical Co., Wilmington, DE). Ang II, Dulbecco's modified Eagle's medium with 25 mM HEPES and 4.5 g/l glucose, actinomycin D, phorbol-12,13-dibutyrate (PDBu), and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Calf serum was purchased from Life Technologies (Rockville, MD). Molecular biology-grade salts and common chemicals were purchased from Sigma Chemical Co., American Bioanalytical Co. (Natick, MA), or Fisher (Pittsburgh, PA). GF 109203X was purchased from Alexis Laboratories (Woburn, MA). [ $\alpha$ - $^{32}$ P]dCTP was obtained from NEN Life Science Products (Wilmington, DE). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA) or Promega (Madison, WI). Agarose was purchased from FMC Bioproducts (Rockland, ME). LB broth, ribonuclease inhibitor, superscript II reverse transcriptase, and *Taq* DNA polymerase were purchased from Life Technologies (Rockville, MD). RNeasy, Oligotex,

TABLE 1  
PCR primers

PCR primers specific for RGS subtypes were designed to anneal to the indicated sequences. The size of the expected product is also indicated.

RGS	Species Accession No.	Primer Sequences 5' to 3'	Expected Product
1	Rat RNU77698	GCCAACCAGATGGGTCAAAATGTC ATGTGGGAGTTGGTGTGTTTGTATCTTC	240bp
2	Mouse MMU67187	AGTAAATATGGGCTGGCTGCATTCA GCCTCTGGATATTTTGGGCAATCA	229bp
	Mouse MMU67187	CGGGAGTCTGAGAATGCAAAGTG CTAGTACTAATGATCTGTAGTCCTAAGAAC	1.1kb
	Rat Our clone	GCCCCATGCTACATGAGACAG TGTAAGTCCCTAAGAAACGTCAGTCAAC	486bp
3	Rat RNU32434	TTTCAAGAAGGTCAAATCGCAGTCCA GTCAAAGCAGCCTCGGGTGAT	162bp
4	Rat RNU32327	TCAGCTGTGAGGAGTACAAGAAAATCAAA TTCTGGGCTTCATCAAAACAGGTTATC	186bp
5	Rat RNU32435	AAGTCCCCCATCAAAATGGCAGAGA CTGGGCCAGGTCAAAGCTGTG	158bp
6	Rat RNU32436	TGGCCGTCCAAGATCTCAAGAAGCAA GTAGATGTGCTCCTGGGCATCTTCAAAT	193bp
7	Rat RNU32328	TTGGCAGTGGAGGACCTGAAGAAAAG CAGCTTGTAATGTGCTCCTGAGCATCT	200bp
8	Rat RNU32432	GCCTGTGAGGAGTTCAAGAAGAC CCCTGGGCTTGATCAAAACAAGTC	184bp
9	Rat RNU32433	CTGAAGTATGGGGATCAGTCCAAGGTCAA TGGGTCTGCGCCGCGTCCAAC	172bp
10	Rat RNU32437	TGAAGATTTCAAGAAAACGGAGGACAAG TTTTTGAACATCAGAGGGTGTGGT	167bp
	Rat AA801173	GGAAGATCCAGAGGGCGTGAAGAGA CACACTCCAGTCTCAAAACACAACAAAG	503bp
11	Rat RNU32438	GTCCCCACCCTGGTGGACT AGCATGTAGATGTGCAGTTGTGCTG	160bp
12	Rat RNU92280	ATCGAAATGTTAGAAAGACCAAAGAGGAC ATGGAAAACCCGGACTTGACAGCA	1.1kb
13	Human AF030107	GGCGGAATTGTTGGATTGTGAAGATGT GAAACTGTTGTTGGACTGCATAGTTTTCA	472bp
14	Rat RNU92279	GGAGGCCCAACATCTACCAT GCATCTTCTACCGACAAACTGGAG	1.1kb
16	Mouse MMU67189	CGAGGAGTTCAAGAAGATCCGATCAG CTGAGCCACATCGAAGCAACTGGT	177bp
	Mouse MMU67189	TCCGCCACCATCCTCTCCAC TGTCGTGAGCCCTCAGTCACAG	512bp
	Rat EST AA817864	AAAACAGGGATTTCGTACACCACTGATAA CGACAACGTAGCAAGGAGAGAAG	423bp
GAIP	Rat AF068136	CCACCCACATGAGGCTGAGAAA GCCCCCTGGAGTAACAGAGAG	627bp

and Qiaquick were obtained from Qiagen (Chatsworth, CA). Advantage cDNA polymerase and KlenTaq DNA polymerase were purchased from Clontech (Palo Alto, CA). The Prime-It II kit was purchased from Stratagene (Menasha, WI). AmpliTaq DNA polymerase FS was obtained from Perkin-Elmer Cetus (Norwalk, CT).

**Cell Culture.** VSMCs were isolated from male Sprague-Dawley rat thoracic aortas through enzymatic digestion as described previously (Griendling et al., 1991). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin and passaged twice a week by harvesting with trypsin/EDTA and seeding into 75-cm<sup>2</sup> flasks. For experiments, cells between passages 6 and 18 were used at confluence. For the calcium deprivation study, HEPES buffer was composed of 150 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, and 20 mM HEPES with and without 1.5 mM CaCl<sub>2</sub>.

**Design of Polymerase Chain Reaction (PCR) Primers.** Reverse transcription (RT)-PCR was used to rapidly identify which RGS sequences are expressed in rat VSMCs. Primers were designed to anneal to known fragments of rat RGS sequences or, when these were unavailable, to mouse (RGS2 and 16) or human (RGS13) sequences. Care was taken to ensure that the 3' end of the primers would differentiate between closely related fragments (e.g., RGS9 and RGS11) as indicated by sequence alignment. Primer sequences for RGS sequences are shown in Table 1.

**RT-PCR.** Total RNA was purified from unstimulated confluent VSMCs with RNeasy spin columns. Poly(A)<sup>+</sup> RNA was obtained by further purification using oligo(dT)-coupled microspheres (Oligotex). cDNA was generated in the presence of ribonuclease inhibitor using an oligo(dT) primer and superscript II reverse transcriptase. PCRs were performed with cDNA primers as described and Taq polymerase in a model 2400 or 9600 thermocycler (Perkin-Elmer Cetus) using the following typical conditions: 95°C for 1 min 45 s and 35 cycles of 95°C for 15 s and 60°C for 1 min/kb of expected product, followed by 72°C for 5 min. The reaction products were analyzed by electrophoresis on agarose gel. PCR products of the expected size were prepared in larger quantities in scaled-up reactions, run on agarose gels, and purified using Qiagen spin columns. Their identity was verified by direct DNA sequencing using dye terminator cycle sequencing with AmpliTaq DNA polymerase FS and a model 373A DNA sequencer (Applied Biosystems, Foster City, CA).

**Preparation of Probes.** To generate an RGS2 probe, PCR was performed with cDNA from rat VSMCs using a second set of primers based on mouse sequence (Table 1) and a mixture of DNA polymerases containing KlenTaq and DeepVent (Advantage cDNA polymerase). The expected 1.1-kb product was cloned and sequenced to confirm its identity. A 486-bp probe was generated by amplification of a plasmid clone using a third set of primers (Table 1). An RGS3 probe was prepared by excision of a 1.6-kb insert from a plasmid with *Hind*III and *Xba*I. The expression plasmid containing human RGS3 (HSU27655) was generously provided by Drs. A. Scheschonka and J. H. Kehrl (National Institutes of Health, Bethesda, MD). A 503-bp RGS10 probe was prepared by PCR amplification of a rat EST (AA801173) generously provided by Dr. M. D. Cotton at The Institute for Genomic Research (Rockville, MD) using primers shown in Table 1. A 1.1-kb RGS12 probe was prepared by PCR amplification of an expression plasmid containing the coding region of rat RGS12 (RNU92280) using primers shown in Table 1. The construct was generously provided by Dr. D. P. Siderovski (Amgen Institute, Toronto, Canada). To prepare GAIP probe, PCR was performed with cDNA from rat VSMCs using primers shown in Table 1 and Taq polymerase. The expected 627-bp product was cloned and sequenced to confirm its identity, and a probe was generated by amplification of a plasmid clone using the same primers.

**RNA Isolation and Northern Blot Analysis.** Total RNA was extracted from cells as described previously (Lassègue et al., 1995). RNA samples (15  $\mu$ g) were separated by electrophoresis in 1.0% agarose gels containing 6.6% formaldehyde. RNA was transferred onto a nylon membrane and immobilized by UV cross-linking (Str-

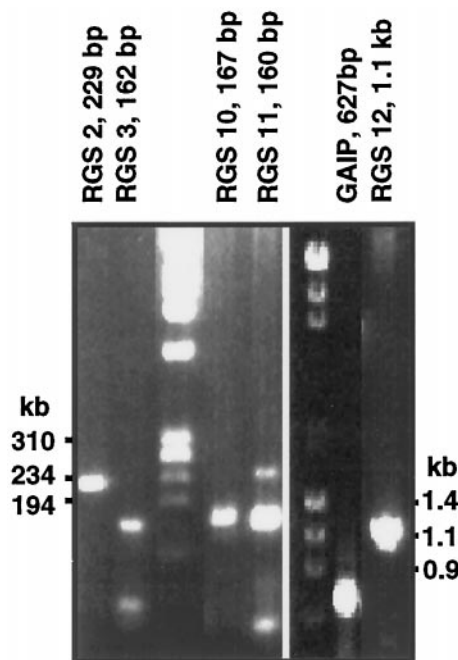
atalinker; Stratagene, La Jolla, CA). The probes for RGS2, 3, 10, and 12 and GAIP were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using a random primer labeling kit (Prime-It II). Membranes were prehybridized at 42°C for 4 h in 1 M NaCl, 0.05 M Tris, pH 7.4, 5 $\times$  Denhardt's solution, 50% formamide, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA. The hybridization was performed overnight at 42°C with <sup>32</sup>P-labeled probe in the same buffer without Denhardt's solution. Membranes were briefly rinsed and washed. For RGS12, RGS3, and GAIP, membranes were washed three times for 15 min in 2 $\times$  SSC plus 0.1% SDS at 55°C. RGS10 and RGS2 membranes were washed three times for 15 min in 1 $\times$  SSC plus 0.1% SDS at 55°C. The hybridization signal was determined using a PhosphorImager (Storm 860; Molecular Dynamics, Sunnyvale, CA) and quantified by densitometry. Staining of the 28S rRNA band by ethidium bromide, after transfer to the membrane, was used for normalization.

**Statistical Analysis.** Results are expressed as mean  $\pm$  S.E. Statistical significance was assessed by ANOVA, followed by comparison of group averages through contrast analysis, using the SuperANOVA statistical program (Abacus Concepts, Berkeley, CA). A *P* value of <.05 was considered to be statistically significant.

## Results

**Expression of RGS mRNAs in VSMCs.** To determine which RGS transcripts were present in VSMCs, RT-PCR primers were designed for RGS1–14 and 16 and GAIP. Of the 16 RGS sequences tested, six (RGS2, 3, 10, 11, and 12 and GAIP) were present in VSMCs, as shown in Fig. 1. The identity of these RGS mRNAs was confirmed by sequencing the PCR product.

**Regulation of RGS Expression by Ang II in Cultured VSMCs.** To determine whether Ang II specifically regulates any of the RGS mRNAs found in VSMCs, cells were stimulated with Ang II (100 nM) for 15 min to 24 h. As shown in Fig. 2, a–d, neither GAIP, RGS3, RGS10, nor RGS12 was significantly regulated by Ang II. For GAIP, there were two



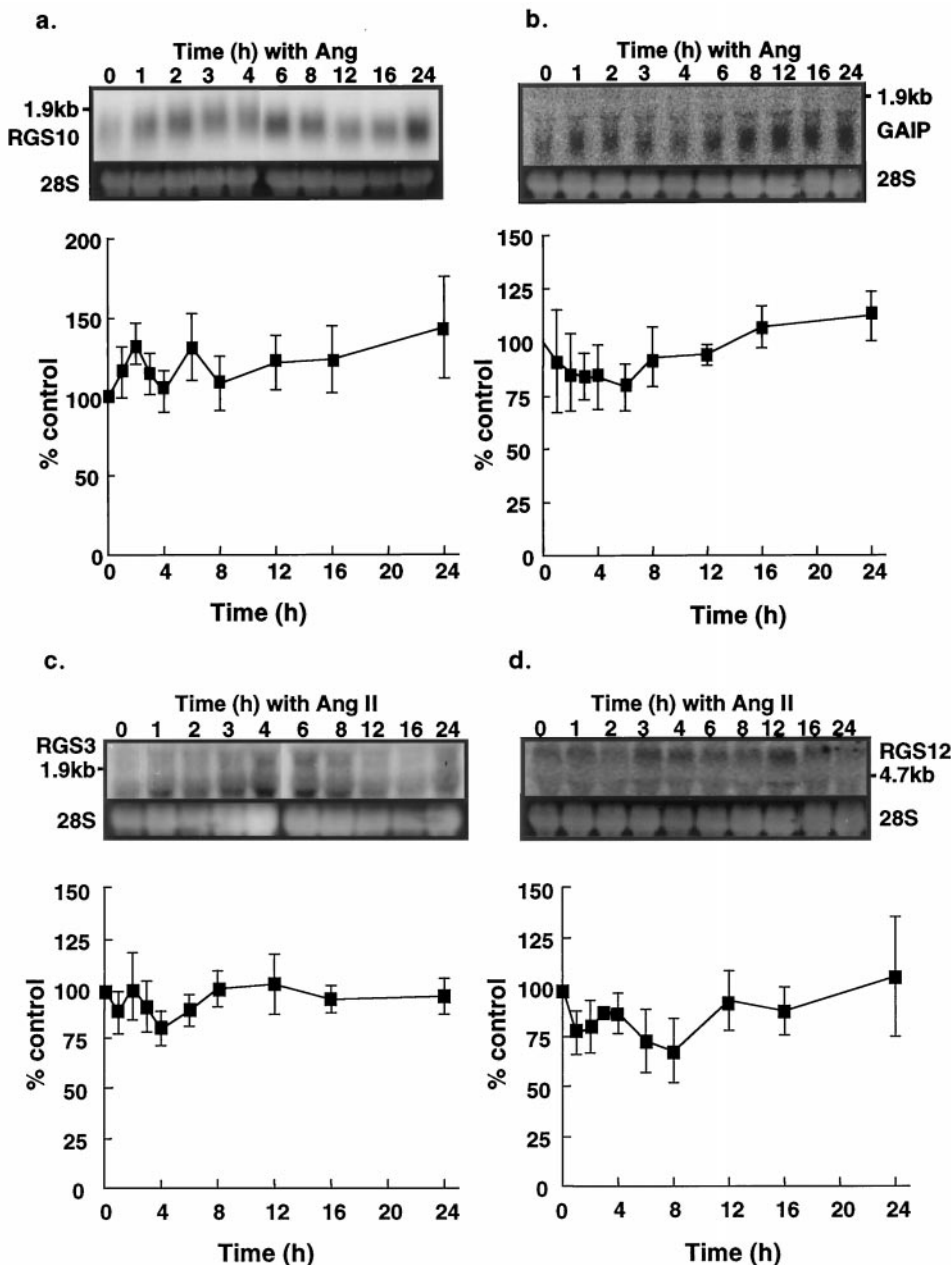
**Fig. 1.** Expression of RGS sequences in VSMCs. PCR primers were designed from known RGS sequences, and RT-PCR was performed. Of the 16 sequences analyzed, only RGS2, 3, 10, 11, 12 and GAIP gave bands of the expected size. The third and sixth lanes represent the appropriate DNA ladders.



transcripts present (~1 and ~1.6 kb). There were also two transcripts for RGS3, at ~3.5 and 1.0 kb, and for RGS12, at ~5.6 and 4.5 kb. In contrast to the other RGS mRNAs, RGS2 transcripts were highly regulated (Fig. 3). After only 15 min of Ang II, both the ~1.8- and ~4.7-kb bands were similarly up-regulated by Ang II. The ~1.8-kb transcript was up-regulated by  $154 \pm 6\%$  of control at 15 min. RGS2 mRNA levels peaked at ~400% of control between 30 min ( $396 \pm 24\%$ ,  $P < .001$ ) and 1.5 h ( $396 \pm 56\%$ ). By 3 h of Ang II stimulation, RGS2 mRNA levels ( $174 \pm 27\%$ ) were not significantly different from control. This up-regulation of RGS2 message by Ang II was dose-dependent (0.1–1000 nM; Fig. 4), with an  $EC_{50}$  of between 1 and 10 nM, corresponding to the  $K_d$  for the  $AT_1R$  of ~2 nM (Murphy et al., 1991). Preincubating the cells with 100  $\mu$ M losartan, a specific  $AT_{1A}R$  antagonist, completely inhibited the Ang II up-regulation of RGS2 at 1 h

(data not shown). Thus, Ang II robustly and selectively up-regulates RGS2 in VSMCs.

**Ang II Signaling Pathway Components Mediating RGS2 Up-Regulation.** The potential involvement of PKC in the regulation of RGS2 was assessed by exposing cells to PMA (100 nM) for 1 to 6 h. As shown in Fig. 5, PMA up-regulated RGS2 within 1 h to  $477 \pm 34\%$  ( $P < .001$ ) of control levels. By 6 h, this response was back ( $124 \pm 33\%$ ) to approximately baseline levels. These observations suggest a role for PKC in the up-regulation of RGS2. To determine whether PKC is involved in agonist-induced regulation, cells were incubated with PDBu (200 nM) for 24 h to down-regulate PKC or GF 109203X (10  $\mu$ M) for 30 min to inhibit PKC. Cells were then exposed to either Ang II (100 nM) or PMA (100 nM) for 1 h. Figure 6 shows that pretreatment with PDBu attenuated the increase in RGS2 after Ang II stimulation by  $48 \pm$

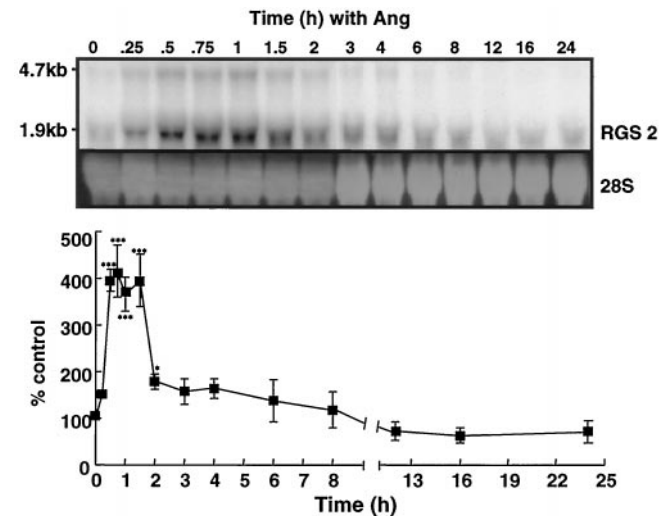


**Fig. 2.** Temporal effect of Ang II on RGS mRNA expression. VSMCs were stimulated with Ang II (100 nM) during a 24-h period. Total RNA was extracted, and Northern hybridization was performed using a cDNA probe for RGS3, 10, and 12 and GAIP. These results are expressed as the mean  $\pm$  S.E. of three experiments, except for RGS3, which was the mean of five experiments. Each point was normalized to 28S rRNA and is expressed as a percentage of control. Top, representative blots. a, RGS10 mRNA, one band at ~1.0 kb; b, GAIP mRNA, two bands under 1.9 kb at ~1.6 and ~1.0 kb; c, RGS3 mRNA, two bands, one at ~3.5 kb and another at ~1.0 kb; d, RGS12 mRNA, two bands, one at ~5.6 kb and one at ~4.5 kb.

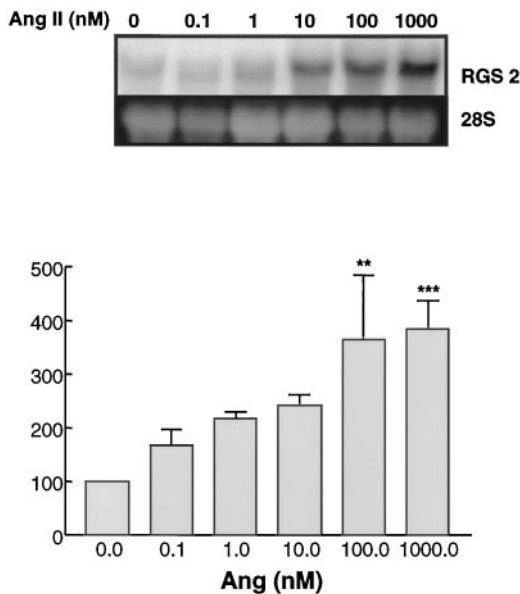
13%. Pretreatment with GF 109203X decreased the baseline of RGS2 by  $35 \pm 5\%$  and attenuated the effect of Ang II ( $50 \pm 4\%$ ) as well. The efficacy of GF 109203X was demonstrated by its ability to completely abolish the effect of PMA on RGS2 message (Fig. 6).

The incomplete effect of PKC inhibition on Ang II-induced RGS2 regulation suggests the involvement of an additional signaling pathway. We therefore tested the potential roles of calcium and tyrosine kinases in this process. As shown in Fig. 7, the tyrosine kinase inhibitor genistein ( $100 \mu\text{M}$ , 30

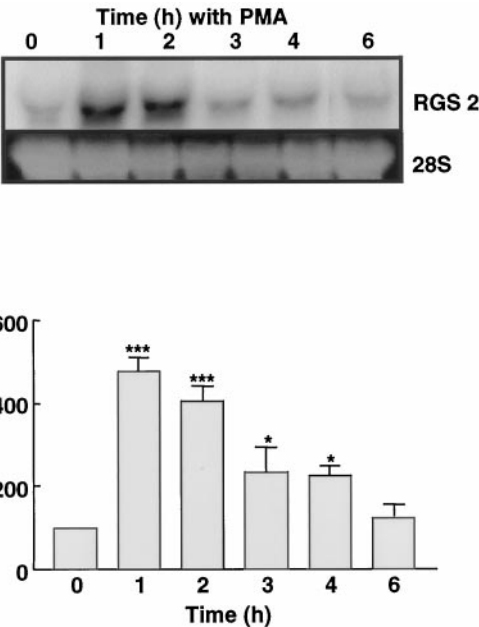
min before the addition of Ang II) had no effect on Ang II stimulation of RGS2. Furthermore, incubation of the cells in a calcium-deprived environment with and without Ang II stimulation for 1 h had no effect on Ang II up-regulation of



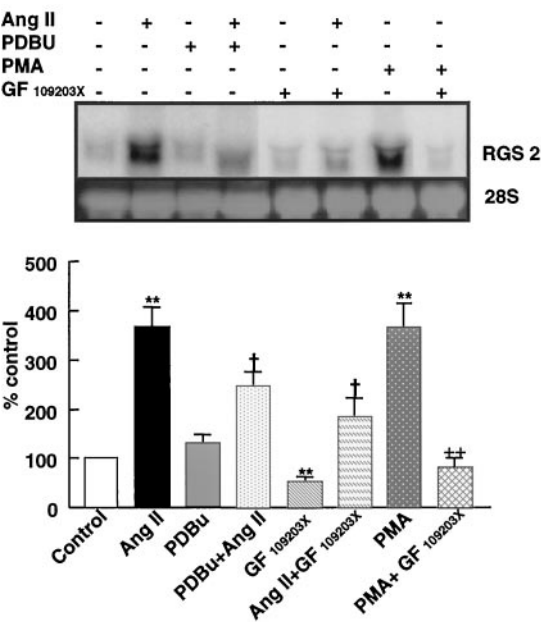
**Fig. 3.** Up-regulation of RGS2 mRNA by Ang II. VSMCs were stimulated with Ang II (100 nM) for 15 min to 24 h. Total RNA was extracted, and Northern hybridization performed using a cDNA probe for RGS2 (486 bp, 3' noncoding region). Points represent the mean  $\pm$  S.E. of four experiments. Each point was normalized to 28S rRNA and is expressed as a percentage of control. Top, shows a representative blot (two mRNA bands,  $\sim 3.7$  and  $\sim 1.8$  kb) and the corresponding 28S rRNA band. The band at  $\sim 1.8$  kb was previously reported as RGS2. \*\*\* $P < .001$ , \*\* $P < .05$  versus control.



**Fig. 4.** Dose dependence of Ang II-induced RGS2 mRNA up-regulation. VSMCs were incubated with 0.1 to 1000 nM Ang II for 1 h. Values are mean  $\pm$  S.E. for four experiments. Each point was normalized to 28S rRNA and is expressed as a percentage of control (dose zero). Top, representative blot ( $\sim 1.8$  kb) and the corresponding 28S rRNA band. \*\*\* $P < .001$ , \*\* $P < .01$  versus control.



**Fig. 5.** RGS2 up-regulation by PMA. VSMCs were stimulated with 100 nM PMA for 1 to 6 h. Values are represented as mean  $\pm$  S.E. for three experiments. Each point was normalized to 28S rRNA and is expressed as a percentage of control (time zero). Top, representative blot with its corresponding 28S RNA band. \*\*\* $P < .001$ , \* $P < .01$  versus control.



**Fig. 6.** Effect of PKC inhibition on Ang II-mediated up-regulation of RGS2. VSMCs were treated with 200 nM PDBu for 24 h to down-regulate PKC, followed by Ang II (100 nM,  $n = 3$ ) for 1 h or PDBu ( $n = 3$ ) or Ang II for 1 h alone ( $n = 7$ ). A separate group of VSMCs was treated with Ang II (100 nM,  $n = 7$ ) or PMA (100 nM,  $n = 5$ ) for 1 h in the presence or absence of GF 109203X (30-min pretreatment,  $10 \mu\text{M}$ ,  $n = 4$ ). Controls were VSMCs incubated with GF 109203X alone ( $n = 4$ ). Each point was normalized to 28S rRNA and is expressed as a percentage of control (time zero). Top, representative blot with the corresponding 28S rRNA band. \*\* $P < .01$  versus control. \* $P < .05$  versus Ang II stimulation. ++ $P < .01$  versus PMA stimulation.

RGS2 in VSMCs. Inhibition of intracellular calcium with the calcium chelator 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester and the calcium ATPase inhibitor thapsigargin did not attenuate the Ang II up-regulation of RGS2 (data not shown).

**Mechanism of RGS2 mRNA Regulation.** To determine whether the up-regulation of RGS2 by Ang II was transcriptionally mediated, cells were incubated with actinomycin D (7.5  $\mu$ g/ml) for 30 min before the addition of Ang II (100 nM) or PMA (100 nM) for 1 h. As shown in Fig. 8a, pretreatment with actinomycin D abolished the up-regulation of RGS2 by both Ang II and PMA, suggesting that this up-regulation is transcriptionally mediated.

A possible contribution of mRNA stabilization to RGS2 up-regulation by Ang II was assessed by incubating cells with Ang II (100 nM) for 1 h to maximally up-regulate the RGS2 message and then exposing them to actinomycin D (7.5  $\mu$ g/ml) for 1 to 3 h. Control cells also received actinomycin D. Ang II stimulation did not appear to stabilize the RGS2 message, as shown in Fig. 8b. The half-life of RGS2 mRNA was  $\sim$ 1 h in the presence or absence of Ang II.

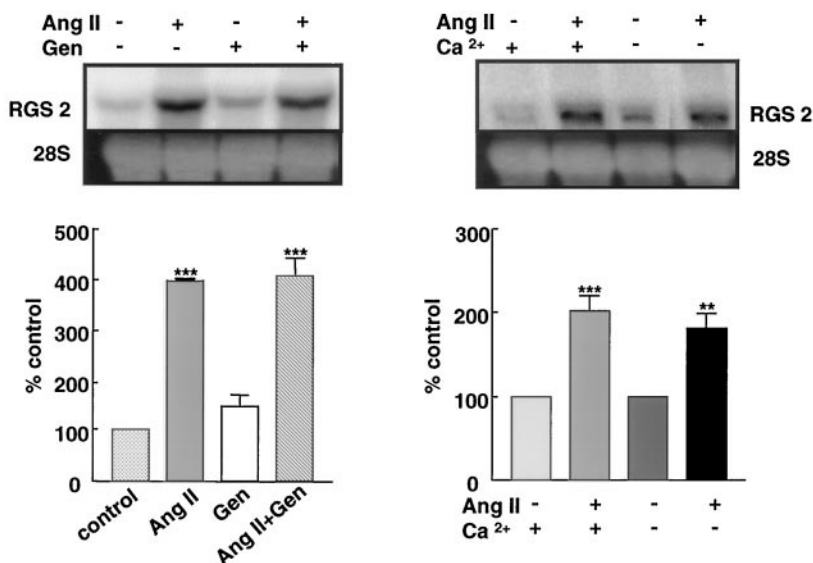
## Discussion

Although there is a rapidly increasing body of knowledge regarding the function of RGS proteins, relatively little is known about regulation at the mRNA level. Only a few investigators have demonstrated that RGS2 mRNA levels can be regulated (Burchett et al., 1998; Ingi et al., 1998). This study is the first to identify which RGS transcripts are present in VSMCs and, most importantly, to show that among these, only RGS2 is specifically regulated by Ang II. Up-regulation of RGS2 mRNA by Ang II is transcriptionally mediated through PKC-dependent and -independent pathways. The identity of the PKC-independent pathway remains to be determined but apparently does not involve tyrosine kinases or calcium.

The newly discovered RGS proteins are a family of proteins that have been shown to be negative regulators of  $G_{\alpha}$  subunit signaling. Considerable evidence indicates that the RGS proteins increase the GTPase activity of the  $G_{\alpha}$  subunits (Berman and Gilman, 1998), thereby inhibiting their function.

RGS2 is one of the members of the RGS family that has been shown to be a selective and potent inhibitor of  $G_{\alpha q}$  (Heximer et al., 1997). RGS2 was also shown, when reconstituted with phospholipid vesicles, to inhibit  $G_q$  activation of PLC $\beta$ 1 (Heximer et al., 1997). RGS2 thus is an attractive candidate to negatively regulate PLC activation by Ang II in VSMCs. Several studies have reported different RGS2 transcript sizes within tissues. Chen et al. (1997) showed two bands in mouse tissues: one at 1.5 kb and another at 1.8 kb. Only in the brain was the larger transcript more abundant than the smaller band. Burchett et al. (1998) showed one RGS2 transcript ( $\sim$ 1.8 kb), as did Ingi et al. (1998). In our study, we observed not only the band at  $\sim$ 1.8 kb but also another transcript at  $\sim$ 4.7 kb. This higher band also appeared to be regulated by Ang II but was not as abundant as the lower transcript.

There has been relatively little published regarding the regulation of RGS expression in general, but several studies have examined RGS2 expression. cAMP-, calcium-, and protein kinase C-dependent pathways have all been found to regulate RGS-2, depending on agonist and cell type. Beadling et al. (1999) demonstrated that RGS2 mRNA was down-regulated in human lymphocytes after interleukin-2 treatment and that this effect was reversed by the addition of dibutyl cAMP, whereas Tseng and Zhang (1998) showed a mild increase in RGS2 mRNA after 60 min of treatment of  $\beta$ TC3 cells with glucose-dependent insulinotropic polypeptide, a cAMP activator. Pepperl et al. (1998) also showed in PC12 cells that forskolin, dibutyl cAMP, and 8-(4-chlorophenylthio)cAMP all increased RGS2 mRNA levels. Although in hepatic cell lines the  $AT_{1A}R$  is known to couple to the  $G_{\alpha i}$  subunit to down-regulate intracellular cAMP levels, in VSMCs there is no evidence that the  $AT_{1A}R$  couples to the  $G_{\alpha i}$  subunit or that it affects cAMP levels (Penit et al., 1983). We have previously shown that both  $G_{\alpha q}$  and  $G_{\alpha 12/13}$  subunits mediate  $AT_{1A}R$  signaling and are upstream of PKC (Ushio-Fukai et al., 1998, 1999). In these cells, PKC appears to be a major regulator of RGS2 expression. In contrast, in human mononuclear cells, a calcium ionophore, but not a PKC activator, increased RGS2 mRNA (Heximer et al., 1997). These results suggest that the RGS proteins may, in



**Fig. 7.** Tyrosine kinase inhibition and calcium deprivation do not affect Ang II-induced RGS2 regulation. Left, VSMCs were treated with Ang II or vehicle for 1 h in the presence or absence of genistein (30-min pretreatment, 100  $\mu$ M). Values are represented as mean  $\pm$  S.E. for four experiments. Each point was normalized to 28S rRNA and is expressed as a percentage of control. Top, representative blot with the corresponding 28S RNA band. Right, cells were incubated with or without calcium in HEPES-balanced salt solution for 30 min and exposed to either vehicle or Ang (100 nM) for 1 h. Values are represented as mean  $\pm$  S.E. for three experiments. Each point was normalized to 28S rRNA and is expressed as a percentage of the appropriate control. Top, representative blot with the corresponding 28S RNA band. \*\*\* $P$  < .01, \*\* $P$  < .01 versus control.



addition to being relatively  $G_{\alpha}$ -specific, be regulated in a cell- and agonist-specific manner.

Recently, Burchett et al. (1998) demonstrated that amphetamine (7.5 mg/kg) injection rapidly but transiently increased RGS2 mRNA in the caudate putamen and the nucleus accumbens. Levels were increased above control by ~1.75-fold within 1 h and returned to baseline by 2 h after amphetamine injection. Ingi et al. (1998) reported that in the rat cerebral cortex, hippocampus, and striatum, RGS2 mRNA was rapidly but transiently up-regulated after electroconvulsive seizure. After the activation of dopamine receptors *in vivo*, RGS2 mRNA levels were induced in the rat striatum within 30 min. The time frame of these *in vivo* results closely resembles that for VSMCs stimulated with Ang II *in vitro*. We demonstrated that RGS2 mRNA was up-regulated by 1.5-fold within 15 min and was back to baseline levels within 6 h after Ang II stimulation. Taken together, these results suggest that RGS2 mRNA expression occurs quite rapidly after receptor stimulation, whether it be  $AT_{1A}$ , muscarinic, or dopamine receptors.

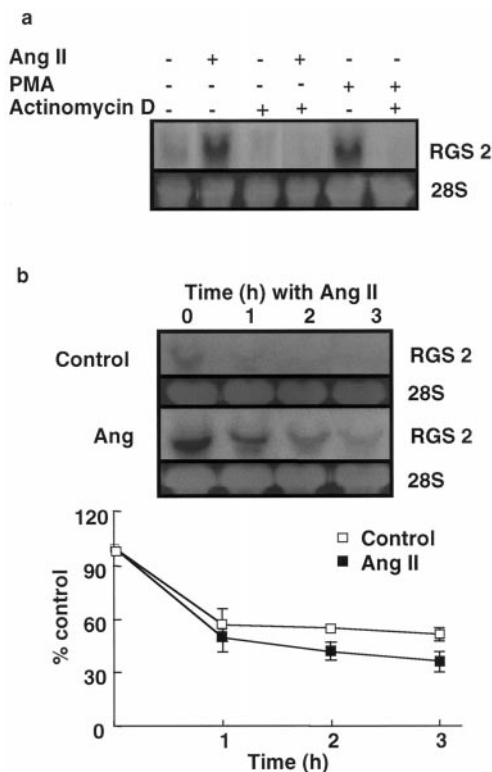
The rapidity with which the message is induced is one of the most striking characteristics of Ang II regulation of RGS2 mRNA. A 30-min stimulation with Ang II is sufficient to cause a maximal up-regulation of RGS2. Our studies with actinomycin D suggest that this effect is transcriptionally mediated and

is not due to stabilization of the message. PMA induction of RGS2 also appeared to depend on enhanced transcription.

The ability of PMA, an activator of PKC, to induce a rapid and profound increase in RGS2 mRNA suggests a role for this kinase in RGS2 regulation. Induction of RGS2 mRNA after Ang II stimulation appeared to be regulated in part by PKC. Two methods of PKC inhibition, down-regulation of PKC with PDBu or inhibition of kinase activity with GF 109203X, only partially attenuated Ang II-induced RGS2 up-regulation. In contrast, PMA-induced RGS2 up-regulation was completely blocked by the PKC inhibitor GF 109293X. GF 109203X, but not PDBu down-regulation, also lowered the baseline levels of RGS2 mRNA expression. This discrepancy between the two methods of PKC inhibition may be due in part to different basal activity of PKC isoforms. Within VSMCs, there are several isoforms of PKC:  $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  (Liao et al., 1997). PDBu has been shown to have no effect on PKC $\zeta$  in VSMCs but to inhibit other PKC isoforms (Liao et al., 1997). In contrast, the concentration of GF 109203X used in this study will inhibit all isoforms of PKC (Martiny-Baron et al., 1993), suggesting that PKC $\zeta$  may contribute to the regulation of basal RGS2 mRNA expression as well.

PKC inhibition did not, however, completely abolish the Ang II-stimulated regulation of RGS2, suggesting that a PKC-independent pathway is also involved. After stimulation of the  $AT_{1A}$ R, many pathways are activated, including PLC $\beta$ , calcium mobilization, and PLD, as well as reactive oxygen species and the mitogen-activated protein kinase signaling cascades (Griendling et al., 1997). Although our data do not conclusively identify the PKC-independent pathway, we have demonstrated that the increase in RGS2 mRNA after Ang II stimulation is not due to activation of the tyrosine kinase pathway and is not calcium-mediated. Further studies are needed to clarify the additional signaling components.

The rapid and robust up-regulation of RGS2 by Ang II suggests that it may play a role in signal transduction. Many of the components of the Ang II signaling pathway have been shown to be regulated at the mRNA level after long-term stimulation. The  $AT_{1A}$ R receptor and  $G_{\alpha q/11}$  are down-regulated by 4 to 6 h of Ang II treatment, whereas G protein-coupled receptor kinase 5 levels are increased after 16 h of Ang II (Lassègue et al., 1995; Kai et al., 1996; Ishizaka et al., 1997). If RGS2 is involved in  $AT_{1A}$ R-mediated effects, one of its actions likely is to terminate  $G_{\alpha q}$  activation of PLC $\beta$ , as has been shown *in vitro* (Heximer et al., 1997). This putative activity would be consistent with our observations that agonist-induced coupling of the  $AT_{1A}$ R to  $G_{\alpha q/11}$  rapidly attenuates as the receptor switches to couple with  $G_{\alpha 12/13}$  (Ushio-Fukai et al., 1998, 1999). Alternatively, like RGS1, 3, and 4, RGS2 may, in addition to exerting GAP activity, increase the availability of the  $G_{\beta\gamma}$  (Doupnik et al., 1997; Bunemann and Hosey, 1998). This would have profound effects on those aspects of Ang II-induced sustained signal generation mediated by  $G_{\beta\gamma}$  (e.g., PLD). RGS2 could also serve a scaffolding function to couple  $AT_{1A}$ Rs to specific G proteins and effectors, as has been suggested for M3 receptors and RGS4 (Zeng et al., 1998). The exact role that RGS2 plays in VSMCs, and in particular in the signaling of G protein-coupled receptors like the  $AT_{1A}$ R, requires an assessment of protein expression and selective inhibition of RGS isoforms. However, until tools are available to address these issues, it is important to note that



**Fig. 8.** Effect of actinomycin D on RGS2 regulation. **a**, VSMCs were incubated with actinomycin D (7.5  $\mu$ g/ml) for 30 min before the addition of Ang II (100 nM) or PMA (100 nM) for 1 h. This gel is representative of two identical experiments. **b**, VSMCs were incubated with either Ang II (100 nM) or vehicle for 1 h, followed by the addition of actinomycin D to all cells. Total RNA was then extracted 1 to 3 h after actinomycin D treatment. Top, representative blot with the corresponding 28S RNA band. Bottom, values are represented as mean  $\pm$  S.E. for three experiments. Each point was normalized to 28S rRNA and is expressed as a percentage of control.

RGS expression is regulated in vivo by the receptors that they may functionally control.

In summary, VSMCs express multiple RGS sequences, but only RGS2 mRNA is stimulated by Ang II. This regulation is both PKC-dependent and -independent and is transcriptionally mediated. This study lends support for the receptor selectivity of the RGS proteins and their involvement in rapid signaling events that occur after G protein-linked receptor activation. Regulation of RGS2 may be of critical importance in modulating the complex signaling events both temporally and spatially that result from AT<sub>1A</sub>R activation.

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