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RGS2 Is a Mediator of Nitric Oxide Action on Blood Pressure and Vasoconstrictor Signaling

Xiaoguang Sun, Kevin M. Kaltenbronn, Thomas H. Steinberg, and Kendall J. Blumer

Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri

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ABSTRACT

The nitric oxide (NO)-cGMP pathway regulates vascular tone and blood pressure by mechanisms that are incompletely understood. RGS2, a GTPase-activating protein for Gqα that is critical for blood pressure homeostasis, has been suggested to serve as an effector of the NO-cGMP pathway that promotes vascular relaxation based on studies of aortic rings in vitro. To test this hypothesis and its relevance to blood pressure control, we determined whether RGS2 functions as an NO effector in smooth muscle of the resistance vasculature. We report that 1) the ability of the NO donor sodium nitroprusside to reduce blood pressure is impaired in RGS2$-/-$ mice, 2) vasopressin-triggered Ca^{2+} transients are augmented in smooth muscle cells from resistance arteries of RGS2$-/-$ mice, and 3) cGMP analogs fail to inhibit vasopressin-triggered Ca^{2+} transients in smooth muscle cells from resistance arteries of RGS2$-/-$ mice even though cGMP-dependent protein kinase (PKG)1α and PKG1β are expressed and activated normally. These results indicated that the NO-cGMP pathway uses RGS2 as a novel downstream effector to promote vascular relaxation by attenuating vasoconstrictor-triggered Ca^{2+} signaling in vascular smooth muscle cells. Genetic or epigenetic impairment of this mechanism may contribute to the development of hypertension, and augmenting it pharmacologically may provide a novel means of treating this disease.

Vascular tone and blood pressure are regulated by the opposing actions of vasoconstrictor and vasodilatory agonists that signal via G protein-coupled receptors (GPCRs) (for reviews, see Carvajal et al., 2000; Lincoln et al., 2001; Munzel et al., 2003). Vasoconstrictors such as angiotensin II, endothelin, vasopressin, and norepinephrine signal via GPCRs coupled to the heterotrimeric G protein Gq. Gq activates signaling cascades in smooth muscle cells that trigger several responses, including Ca^{2+} release from intracellular stores and Ca^{2+} entry across the plasma membrane, resulting in myosin light chain phosphorylation and contraction.

Vasoconstrictor signaling and contraction are potently antagonized by vasodilatory agonists such as acetylcholine, which signal via GPCRs in endothelial cells, producing nitric oxide (NO) and other vasoactive substances that relax smooth muscle cells. NO relaxes smooth muscle by activating guanylyl cyclase, producing cGMP, and activating type I cGMP-dependent protein kinases (PKG1 or cGK1). Activated PKG1 exerts its effects in part by blunting vasoconstrictor-triggered Ca^{2+} transients (Felbel et al., 1988; Francis et al., 1988; Lincoln et al., 1990; Pfeifer et al., 1998) and impairing Ca^{2+} sensitivity of the contractile apparatus (Kitazawa et al., 1991; Lee et al., 1997; Somlyo and Somlyo, 2000). PKG1 attenuates Ca^{2+} transients by several mechanisms, including 1) phosphorylation and activation of IRAG, an inhibitory subunit of the inositol (1,4,5)-trisphosphate (IP_3) receptor (Ammendola et al., 2001; Schlossmann et al., 2000); 2) phosphorylation and inactivation of phospholamban (Raeymaekers et al., 1988; Cornwell et al., 1991), the inhibitory subunit of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase that clears Ca^{2+} from the cytoplasm; and 3) phosphorylation and activation of BK_{Ca} channels (Alioua et al., 1998; Fukao et al., 1999), thereby hyperpolarizing the plasma membrane and blunting Ca^{2+} entry via L-type channels.

PKG1 also attenuates early events in GPCR-activated Ca^{2+} signaling pathways, including GPCR-stimulated phospholipase C activation and IP_3 accumulation (Hirata et al.,

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ABBREVIATIONS: GPCR, G protein-coupled receptor; NO, nitric oxide; PKG1, type I cGMP-dependent protein kinase; IRAG, inhibitory subunit of the inositol (1,4,5)-trisphosphate receptor; IP_3, inositol (1,4,5)-trisphosphate; RGS, regulator of G protein signaling; HR, heart rate; MAP, mean arterial pressure; PE, phenylephrine; SNP, sodium nitroprusside; DMEM, Dulbecco's modified Eagle's medium; VSMC, vascular smooth muscle cell; 8-Br-cGMP, 8-bromo-cGMP; 8-pCPT-cGMP, 8-(*p*-chlorophenylthio)-cGMP; ROI, region of interest; VASP, vasodilator-stimulated phosphoprotein; AT1, angiotensin II type I; AVP, arginine-8-vasopressin; A-VSMC, aortic-vascular smooth muscle cell; MA-VSMC, mesenteric-vascular smooth muscle cell.

1990; Lincoln and Cornwell, 1993; Azula et al., 1996). Inhibition of these early signaling mechanisms that otherwise are responsible for amplifying GPCR-generated signals may provide a particularly important mechanism by which cGMP attenuates Ca^{2+} signaling and promotes relaxation. However, this hypothesis has not been tested. Likewise, the mechanisms by which cGMP inhibits GPCR signaling pathways leading to IP_3 production are poorly understood (for review, see Carvajal et al., 2000). However, evidence suggests that PKG1 can phosphorylate phospholipase Cβ (Xia et al., 2001) and receptors (Wang et al., 1998), potentially uncoupling them from G proteins.

Several observations suggested that RGS2 may function as a novel target or effector of the NO-cGMP pathway that controls blood pressure and vascular tone by regulating vasoconstrictor signaling. RGS2 is one of ~30 regulator of G protein signaling (RGS) proteins that attenuate signaling by stimulating the ability of G protein α subunits to hydrolyze GTP (for reviews, see Ross and Wilkie, 2000; Hollinger and Hepler, 2002), returning them to the inactive GDP-bound state. We have shown that RGS2−/− mice are markedly hypertensive (Heximer et al., 2003). Furthermore, RGS2 binds to and is phosphorylated by PKG1α in vitro and in cells, apparently increasing the ability of RGS2 to associate with membranes and stimulate GTP hydrolysis by Gqα (Tang et al., 2003), the activator of phospholipase Cβ and preferred substrate of RGS2. Aortic rings from RGS2−/− mice exhibit augmented contraction and impaired cGMP-mediated relaxation in vitro (Tang et al., 2003). However, the significance of these in vitro studies for blood pressure homeostasis is not clear because the aorta does not have a major role in blood pressure regulation, and impaired NO- or cGMP-mediated relaxation of the aorta was observed only when endothelium was denuded (Tang et al., 2003). Whether RGS2 regulates contraction or relaxation of smooth muscle of the resistance vasculature, which critically determines blood pressure, is an important problem that has not been investigated. Likewise, the vascular smooth muscle signaling mechanisms that are altered in cells lacking RGS2 are poorly understood.

To identify physiological and cellular signaling mechanisms by which RGS2 regulates blood pressure, we have determined whether this regulatory protein functions in smooth muscle of the resistance vasculature as an effector of the NO-cGMP pathway that promotes relaxation. We have investigated whether RGS2 mediates the ability of an NO donor that acts directly on smooth muscle to reduce blood pressure and the ability of cGMP analogs to attenuate vasoconstrictor-induced Ca^{2+} signaling in resistance artery smooth muscle cells in vitro. The results provide the first evidence indicating that RGS2 mediates the action of the NO-cGMP pathway on blood pressure by promoting relaxation of the resistance vasculature through its ability to attenuate vasoconstrictor-induced Ca^{2+} signaling.

Materials and Methods

Mice and Blood Pressure Measurements. All procedures using mice were performed in strict accordance with protocols approved by the Washington University School of Medicine Animal Studies Committee. Wild-type, RGS2+/−, and RGS2−/− mice backcrossed >10 generations into the C57BL/6 background were used in this study (Oliveira-Dos-Santos et al., 2000; Heximer et al., 2003). Male RGS2+/+ and −/− mice aged 3 to 5 months were obtained by crossing RGS2+/− mice. For measuring blood pressure, mice were anesthetized with a 1.5% isoflurane/oxygen mixture administered with a vaporizer (Ohmeda Isotec 3). Isoflurane was used because it caused the least depression of heart rate compared with other anesthetics [ketamine/xylazine, 87 and 13.4 mg/kg i.p., respectively), urethane (1500 mg/kg i.p.), Avertin (250 mg/kg i.p.), or metofane inhalation (data not shown; see Lorenz, 2002)]. Depth of anesthesia was assessed by lack of response to a pinch. As described in our previous study (Heximer et al., 2003), we recorded heart rate (HR) and mean arterial pressure (MAP) by catheterizing the right common carotid artery with a Millar 1.4 French blood pressure probe and transducer (Millar Instruments Inc., Houston, TX) coupled to a Powerlab/4sp data acquisition system (ADInstruments Pty Ltd., New Castle, Australia) and passing the probe into the ascending aorta. All vasoactive drugs (Sigma-Aldrich, St. Louis, MO) diluted in 0.9% NaCl were administered intravenously by catheterization of the left jugular vein with polyethylene-10 tubing attached to a 0.5-ml syringe. Drugs were administered continuously via an infusion pump (KD Scientific, Holliston, MA) as 20-μl doses added continuously at a rate of 2 μl/s or as a bolus dose of 5 μl added over 3 to 5 s. Saralasin (40 μg/kg/min; Ullman, 1999) was infused intravenously for 10 min to block angiotensin receptors and reduce blood pressures in wild-type and RGS2−/− mice to similar levels. After equivalent baseline blood pressures were established, we administered to each mouse a series of bolus injections containing phenylephrine [PE; 25 μg/kg i.v., which increased MAP to 70% of the value obtained with a maximal dose of PE (Heximer et al., 2003)] alone or in combination with various amounts of sodium nitroprusside (SNP): PE alone, PE + 15 μg/kg SNP, and PE + 45 μg/kg SNP. These are the lowest effective doses of SNP as established previously (Holschneider et al., 2002). After a given injection exerted its effects on blood pressure and blood pressure values returned to starting baseline values, a subsequent injection was administered. After the effects of the last PE + SNP injection waned, we administered a final dose of PE alone (25 μg/kg) and recorded the resultant pressor response. Because this pressor response was indistinguishable in magnitude and kinetics relative to the pressor response elicited by PE at the beginning of the experiment (data not shown), we concluded that the intervening administration of PE + SNP did not damage the resistance vasculature or heart. Whether such damage occurred potentially was a concern because SNP can be metabolized into superoxide or peroxynitrite, which, if present long enough, can trigger apoptosis of smooth muscle cells (for review, see Yamamoto and Bing, 2000). Saralasin (60 μg/kg) was administered between injections as needed to maintain angiotensin II type I (AT1) receptor blockade.

Cell Culture and Ca^{2+} Signaling. Vascular smooth muscle cells were isolated from thoracic aortas of age-matched (4 months), wild-type, and RGS2−/− mice as described previously (Heximer et al., 2003). In brief, freshly isolated aortas were digested with collagenase [0.07% (w/v)] and elastase [0.03% (w/v)] with gentle agitation for 30 min at 37°C in DMEM digestion buffer. Dissociated cells were collected by centrifugation and seeded on poly-L-lysine-coated coverslips. Cells were grown 3 to 5 days in DMEM/F12 media containing 20% fetal calf serum and 2 mM glutamine with penicillin and streptomycin. Smooth muscle cells from mesenteric resistance arteries were isolated and cultured as described previously (Dubey and Overbeck, 1994; Rodrigo et al., 2002). In brief, after mice were euthanized by CO_2 inhalation, a midline incision was made and the mesenteric arcade containing arteries and veins was exposed. Superficial veins and fatty tissue were removed to expose resistance arteries (diameter ~100 μm). Resistance arteries were excised and incubated in DMEM. After remaining fatty tissue and blood clots were removed, arteries were cut into small pieces and placed between one coverslip treated with 0.5% poly-L-lysine and another untreated coverslip in a 24-well plate. Cells were cultured 7 to 10 days in DMEM/F12 containing 20% fetal bovine serum, 20 ng/ml platelet-derived growth

factor, 2 mM glutamine, penicillin, and streptomycin. Staining with anti-smooth muscle α-actin antibody revealed that aortic and mesenteric artery cultures contained >90% smooth muscle cells (data not shown).

For Ca^{2+} signaling experiments, vascular smooth muscle cells from aorta or mesenteric resistance arteries were washed and incubated in 4 μM Fura-2/acetoxymethyl ester (Molecular Probes, Eugene, OR) in Ca1 solution (11 mM glucose, 130 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 17 mM HEPES, and 1 mM $CaCl_2$, pH 7.3) for 30 min at 37°C. Cells were washed once and incubated in Ca1 without Fura-2 for 10 min at 37°C to allow hydrolysis of the acetoxymethyl ester. Coverslips were placed in a RC-26G large open-bath recording chamber (Warner Instrument, Hamden, CT) mounted on a Nikon Eclipse E600 FN microscope equipped with a Nikon Fluor 40×/0.80 water immersion lens (Nikon, Melville, NY). Excitation light (340/380 nm) was provided by a TILL Polychrome IV monochrometer (TILL Photonics GmbH, Martinsried, Germany) in conjunction with a 495-nm dichroic mirror and a 525 ± 20-nm emission filter (Chroma Technology Corp., Brattleboro, VT). To activate PKG1 in vascular smooth muscle cells (VSMCs), cells were mounted in the recording chamber as described above and treated for 10 min with 8-Br-cGMP (1 mM) or 8-pCPT-cGMP (100 μM). During subsequent stimulation with agonists, 8-Br-cGMP (1 mM) or 8-pCPT-cGMP (100 μM) was also present. Fluorescence ratio imaging was performed with TILLvisION 4.0 imaging software (TILL Photonics GmbH), and images were acquired with a SensiCam cooled charge-coupled device camera (PCO Computer Optics GmbH, Kelheim, Germany). Regions of interest (ROI) contained 10 to 20 VSMCs. Image pairs of the ROI were taken every 2s and background-corrected ratio images (340 nm/380 nm) were obtained. Data for each ROI were collected as relative ratio values over time. Ratio data were converted into intracellular calcium concentration ($[Ca^{2+}]_i$) by using a standard look-up table created with a Ca^{2+} calibration kit (Molecular Probes).

Immunoblotting. Aortas were minced and incubated for 30 min with or without 8-pCPT-cGMP (100 μM) in DMEM/F12 at 37°C with 95% O_2/5% CO_2 and snap frozen in liquid nitrogen. Samples were thawed and homogenized with preheated (95°C) Laemmli sample buffer containing SDS. After extracts were centrifuged, the supernatant fractions were collected. Samples (20 μg protein/lane) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked 1h at room temperature in phosphate-buffered saline-0.1% Tween 20 containing 5% fat-free dried milk and incubated overnight at 4°C with primary antibody [anti-PKG1α or -PKG1β antibodies; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; diluted 1:300 in phosphate-buffered saline-0.1% Tween 20 + 1% milk; anti-vasodilator-stimulated phosphoprotein (VASP), diluted 1:500, or anti-VASP monoclonal antibodies specific for phosphoserine-157 or phosphoserine-239, diluted to 0.5 μg/ml; Alexis, San Diego, CA] (Smolenski et al., 1998). Blots were washed and incubated 1 h with horseradish peroxidase-conjugated secondary antibodies (diluted 1:3000; MP Biomedicals, Irvine, CA) followed by enhanced chemiluminescence detection.

Statistics. Student's t test (two-tailed, two sample, equal variance) was used to determine whether differences in the values of blood pressure or $[Ca^{2+}]$ obtained with wild-type and RGS2−/− mice or cells were statistically significant ($p < 0.05$).

Results

RGS2−/− Mice Exhibit Impaired Blood Pressure Response to Sodium Nitroprusside. To determine whether RGS2 promotes NO-mediated relaxation of smooth muscle in the resistance vasculature, we used the following procedure in which blood pressure recordings were used to assess contraction and relaxation of the resistance vasculature independent of the endothelium in wild-type and RGS2−/− mice. To record blood pressure and heart rates, we inserted a pressure-transducing catheter into the right common carotid artery and passed it to the ascending aorta of mice anesthetized with isoflurane (Lorenz, 2002). The left jugular was catheterized with polyethylene-10 tubing to administer vasoactive compounds. Because RGS2−/− mice exhibit elevated blood pressure that depends nearly entirely on signaling by AT1 receptors, we infused an angiotensin II receptor blocker (saralasin, a weak partial agonist; 40 μg/kg/min i.v. for 10 min) to produce similar blood pressures in wild-type and RGS2−/− mice (MAP ~70 mm Hg; Fig. 1A). Because saralasin has a half-life of a few minutes, we administered additional doses of this drug as required in subsequent experiments to maintain baseline MAP values.

To verify that mice responded to vasoactive substances under these conditions, we administered a single bolus injection of PE (25 μg/kg i.v.) that stimulates contraction of the resistance vasculature by activating Gq-coupled α1-adrenergic receptors and followed changes in blood pressure (MAP) and HR. The results shown in Fig. 1B indicated that wild-type and RGS2−/− mice responded similarly to PE challenge. After PE administration, blood pressure in wild-type and RGS2−/− mice quickly rose to similar levels and then declined to baseline values within ~2 min. As expected for mice with functional baroreflexes, PE infusion caused HR to change inversely with respect to MAP. The similar effects of PE on MAP and HR in wild-type and RGS2−/− mice are consistent with results of our previous dose-response experiments showing that increases in blood pressure elicited by various doses of PE are not affected by the loss of RGS2 (Heximer et al., 2003). Thus, there was no evidence suggesting that PE-induced contraction of the resistance vasculature was augmented in RGS2−/− mice, in contrast to the augmentation of PE-induced contraction of aortic rings from RGS2−/− mice observed in vitro (Tang et al., 2003).

To determine whether RGS2 mediates the ability of the NO pathway to relax the resistance vasculature and decrease blood pressure, we prepared saralasin-treated mice as described above and then administered a bolus injection of PE (25 μg/kg, as before) that contained either of two concentrations (15 or 45 μg/kg i.v.) of the short-lived NO donor SNP. Controls with wild-type mice indicated that SNP attenuated the initial PE-induced pressor response (i.e., maximal rise in MAP was reduced relative to PE-only controls), and then drove blood pressure to levels below starting baseline values (Fig. 1, C and D). Owing to the short lifetime of SNP in the circulation, these vasodilatory responses were transient as indicated by the return of blood pressure to baseline values within ~90 s.

Results obtained from identical experiments performed with RGS2−/− mice indicated that SNP-induced vasodilatation was significantly impaired (Fig. 1, C and D). At either a low or high dose of SNP, the vasodilatory effect observed was reduced in magnitude (i.e., MAP declined less in RGS2−/− mice) and duration (i.e., after reaching its low point, MAP in RGS2−/− mice returned to baseline values more rapidly). In contrast, during these experiments heart rates were indistinguishable between wild-type and RGS2−/− mice, thereby ruling out one potential cause for differences in SNP-induced blood pressure responses. The impairment of SNP-triggered blood pressure responses in RGS2−/− mice were unlikely to be caused by differences in blood volume regulation because

the effects of SNP occurred over short time scales (<2 min) and were rapidly reversible. Therefore, the diminished effects of SNP on blood pressure regulation in RGS2−/− mice were indicative of impaired relaxation of the resistance vasculature. However, SNP action was not completely blocked in RGS2−/− mice, consistent with the hypothesis that the NO-cGMP pathway uses RGS2 and other targets to promote relaxation.

Augmented Vasoconstrictor-Induced Ca^{2+} Signaling in VSMCs from RGS2 Knockout Mice. The preceding results supported the hypothesis that RGS2 is an important effector of the NO-cGMP pathway in smooth muscle that promotes vascular relaxation. To test this hypothesis directly, we investigated whether RGS2 regulates smooth muscle cell signaling pathways that control contraction or relaxation. This hypothesis has not been examined previously, although studies have shown that overexpression of RGS2 strongly inhibits signaling by various Gq-coupled receptors (Heximer et al., 1997, 1999), and overexpression of the domain of RGS2 that binds PKG1α inhibits the ability of cGMP analogs to attenuate Gq signaling in fibroblasts (Tang et al., 2003). Although the latter result suggested that RGS2 is a target of PKG1, it did not address what occurs in vascular smooth muscle cells or whether RGS2 has a major or minor role mediating the effects of PKG1. These uncertainties remained because overexpression of the PKG1 binding domain of any target protein could block many or all the effects of this protein kinase. Analyzing cells lacking RGS2 provides a clearer means of addressing these questions.

To determine whether RGS2 regulates signaling in VSMCs, we analyzed the ability of various agonists that signal via Gq-coupled receptors to trigger Ca^{2+} transients in VSMCs from wild-type and RGS2−/− mice. Experiments were performed with primary VSMCs from both aorta and mesenteric resistance arteries isolated, cultured, and loaded with Fura-2 as described under *Materials and Methods*.

First, we screened various GPCR agonists for those that elicited robust Ca^{2+} transients in the majority of cells. The results of these Ca^{2+} imaging experiments performed in the presence of extracellular Ca^{2+} indicated that arginine-8-

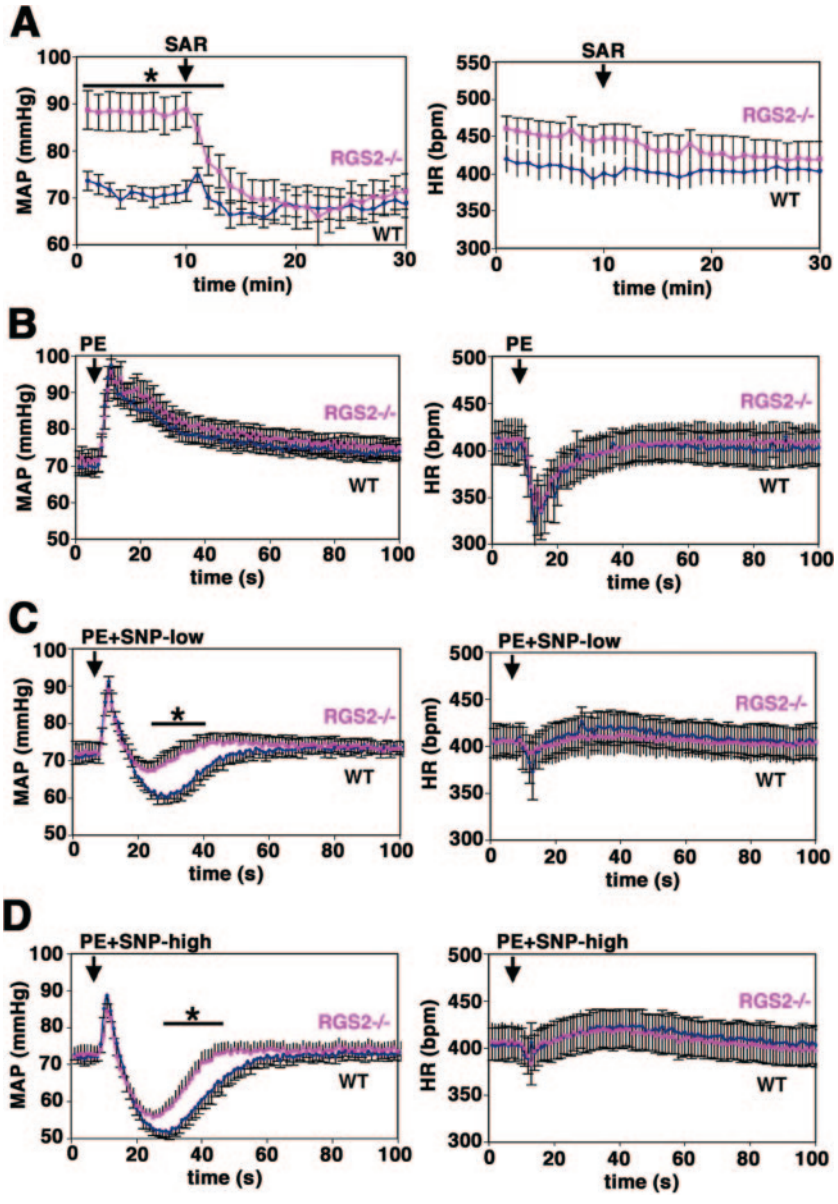

Fig. 1. RGS2 mediates the ability of an NO donor to reduce blood pressure. The ability the NO donor SNP to antagonize the pressor effects the α1-adrenergic receptor agonist PE was used to assess relaxation of the resistance vasculature in wild-type and RGS2−/− mice (n = 7 wild type; n = 8 RGS2−/−; S.E.M. indicated by error bars). A, reversal of the hypertensive phenotype of RGS2−/− mice upon infusion of the AT1 receptor blocker saralasin (40 μg/kg/s i.v.). Saralasin treatment resulted in equivalent baseline blood pressures and heart rates in wild-type and RGS2−/− mice. The series of data points significantly ($p < 0.05$) different between wild-type and RGS2−/− mice is marked with an asterisk (*) over a line. B, blood pressure and heart rate responses of saralasin-treated mice after a single injection of the α1-adrenergic agonist PE (25 μg/kg i.v.). PE administered at the indicated time point elicited equivalent blood pressure and heart rate responses in wild-type and RGS2−/− that were indicative of a functional resistance vasculature and baroreflex. C and D, blood pressure and heart rate responses of saralasin-treated mice after injection of PE (25 μg/kg i.v.) containing a low (15 μg/kg i.v.) or high (45 μg/kg i.v.) dose of SNP. Blood pressure responses elicited by SNP were significantly impaired ($p < 0.05$ as indicated by data points marked with a line and *) in RGS2−/− mice, whereas the effect of SNP on heart rate was not significantly different between wild-type and RGS2−/− mice.

vasopressin (AVP, a vasopressin receptor agonist) and UTP (a P2Y receptor agonist), which signal via receptors coupled to Gq (the preferred substrate of RGS2; Heximer et al., 1999), elicited robust increases in $[Ca^{2+}]_i$ in >90% of aortic or mesenteric resistance artery VSMCs (A-VSMCs or MA-VSMCs, respectively; data not shown). In contrast, phenylephrine or angiotensin II elicited weaker or heterogeneous responses (~30% of cells responded; data not shown), indicating that receptors for these agonists were less well or heterogeneously expressed. Subsequent experiments therefore used A-VSMCs and MA-VSMCs stimulated with AVP, an agonist relevant to blood pressure control, or UTP, to determine whether the effects of RGS2 were receptor-specific. Equivalent results were obtained with both agonists (data not shown), consistent with previous studies indicating that, unlike several other RGS proteins, RGS2 does not seem to exhibit receptor-specific regulation of Gq signaling (Xu et al., 1999; Wang et al., 2004). Therefore, only data obtained with AVP are presented below.

Figure 2 shows results indicating that the potency and efficacy of AVP were augmented in A-VSMCs and MA-VSMCs from RGS2−/− mice. When stimulated by a maximally effective dose of AVP, A- and MA-VSMCs from RGS2−/− mice exhibited peak Ca^{2+} transients that were 20 to 30% higher than obtained with wild-type cells (Fig. 2, A and E). By normalizing the data to the maximal peak value, we noted that the Ca^{2+} transients were more sustained in A- and MA-VSMCs from RGS2−/− mice (Fig. 2, B and F). By measuring peak Ca^{2+} responses elicited by various concentrations of AVP, we found that the potencies of these agonists were augmented 2- to 4-fold in A- and MA-VSMCs from RGS2−/− mice relative to wild-type controls (Fig. 2, C, D, G, and H). These effects were not caused by changes in intracellular Ca^{2+} pools, because similar Ca^{2+} responses were observed with wild-type and RGS2−/− A- and MA-VSMCs treated with ionomycin in the absence of extracellular Ca^{2+} (results presented in next section). Therefore, augmentation of AVP-stimulated Ca^{2+} signaling in RGS2−/− cells may be caused by more complete release of Ca^{2+} from intracellular stores, augmented influx of Ca^{2+} from the medium and/or attenuated Ca^{2+} reuptake into endoplasmic reticulum stores. In contrast to these findings, we previously showed that loss of RGS2 does not significantly augment vasoconstrictor-triggered rises in blood pressure (Heximer et al., 2003), possibly because loss of RGS2 does not augment activation of RhoA or other molecules that promote contraction. Although the present study was not designed to address such questions, the results of the Ca^{2+} signaling experiments presented above established a system that enabled us to address whether the NO-cGMP pathway uses RGS2 to attenuate vasoconstrictor-induced Ca^{2+} signaling.

RGS2 Mediates the Ability of cGMP Analogs to Attenuate Vasoconstrictor-Induced Ca^{2+} Transients. A principal means by which the NO-cGMP pathway inhibits contraction and causes relaxation is by blunting Ca^{2+} transients triggered by vasoconstrictors. RGS2 could be an especially important effector of the NO-cGMP pathway because it is a powerful inhibitor of Gq, the G protein that activates phospholipase Cβ to produce the second messenger IP_3 responsible for triggering release of Ca^{2+} from intracellular stores. RGS2 therefore is poised to regulate the amplification of receptor-generated Ca^{2+} signals, possibly by serving as a target or effector of the NO-cGMP pathway.

To determine whether RGS2 mediates the ability of cGMP to attenuate vasoconstrictor-induced Ca^{2+} transients, we performed Ca^{2+} imaging experiments with wild-type and RGS2−/− A- and MA-VSMCs that were treated with or without 8-Br-cGMP (1 mM) or 8-pCPT-cGMP (100 μM) to activate endogenously expressed cGMP-dependent protein kinases (PKG1) and then stimulated with AVP or UTP. Both cGMP analogs were used to ensure that the effects observed were caused by activation of PKG1 rather than inhibition of phos-

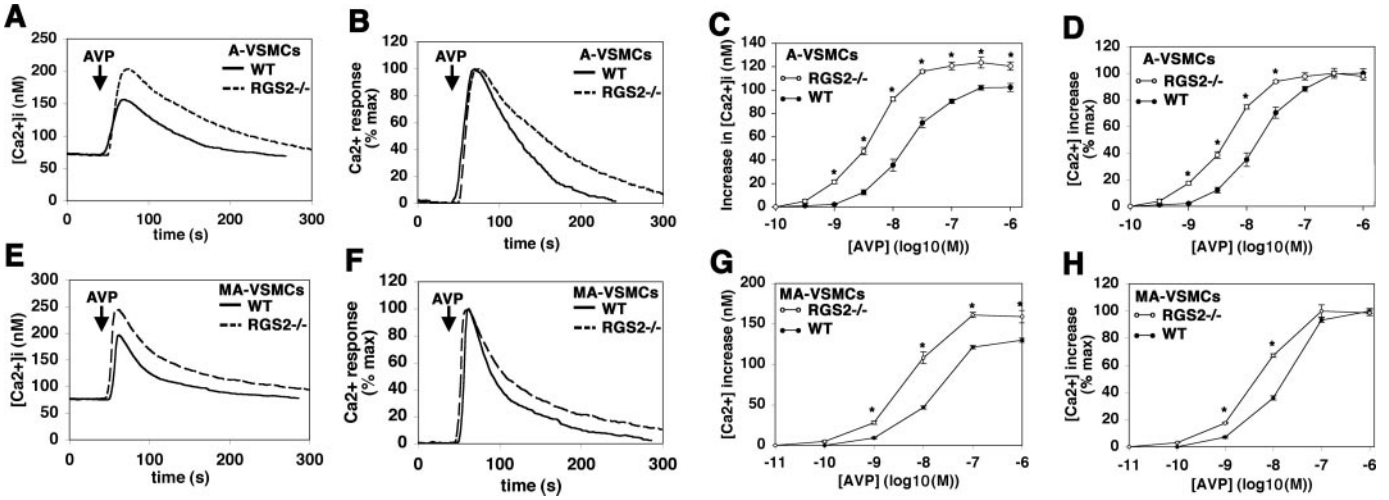

Fig. 2. RGS2 regulates signaling by Gq-coupled vasopressin receptors in aortic and mesenteric resistance artery vascular smooth muscle cells. Increases in $[Ca^{2+}]_i$ elicited by the vasopressin receptor agonist AVP were recorded in Fura-2-loaded primary smooth muscle cells from aorta (A-VSMCs) or mesenteric resistance arteries (MA-VSMCs) from wild-type and RGS2−/− mice. Results from experiments using AVP were obtained in the continued presence of extracellular Ca^{2+}. Fura-2 fluorescence data recorded continuously before and after stimulation were converted to $[Ca^{2+}]_i$ with the use of lookup tables generated with a $[Ca^{2+}]$ calibration kit. A, B, E, and F show average (n = 3–5) time courses of wild-type and RGS2−/− cells (10–20 cells/field/experiment) stimulated with AVP (100 nM). C, D, G, and H show the magnitude of peak Ca^{2+} responses of wild-type and RGS2−/− cells stimulated by various concentrations of AVP. Results of dose-response experiments are the average of three to five independent experiments; error bars indicate S.E.M. Statistically significant ($p < 0.01$) differences in the dose-response curves obtained with wild-type and RGS2−/− cells are indicated by an asterisk (*).

phodiesterase activity that could raise levels of both cGMP and cAMP and thus potentially make it difficult to determine which second messenger system was operative (Butt et al., 1992). Because equivalent results were observed with both cGMP analogs and receptor agonists (data not shown), only the results of experiments using 8-pCPT-cGMP and AVP are presented below.

The results of Ca^{2+} imaging experiments revealed a striking difference in the ability of wild-type and RGS2−/− VSMCs to respond to cGMP analogs. As expected, treatment of wild-type A- or MA-VSMCs with 8-pCPT-cGMP significantly attenuated peak Ca^{2+} signals elicited by a maximally effective concentration of AVP (Fig. 3A). In contrast, treatment of RGS2−/− A- or MA-VSMCs with 8-pCPT-cGMP had minimal inhibitory effect on peak AVP-triggered Ca^{2+} transients (Fig. 3B). This effect was not caused by differences in the amount of Ca^{2+} in intracellular stores, because ionomycin elicited similar Ca^{2+} responses in wild-type and RGS2−/− cells pretreated or not with 8-pCPT-cGMP in the absence of extracellular Ca^{2+} (Fig. 4).

To quantify the inhibitory effects cGMP analogs on AVP-triggered Ca^{2+} responses in wild-type and RGS2 −/− VSMCs, we calculated the integrated areas under Ca^{2+} response curves. Results shown in Fig. 4 indicated that in wild-type cells 8-pCPT-cGMP attenuated AVP-triggered Ca^{2+} responses by >60%. In contrast, in RGS2−/− VSMCs 8-pCPT-cGMP had little inhibitory effect (<5%) on AVP-elicited Ca^{2+} responses. The greatly diminished inhibitory effect of 8-pCPT-cGMP in RGS2−/− cells was not receptor-specific because similar results were obtained by comparing the integrated UTP-stimulated Ca^{2+} signals elicited in wild-type and RGS2−/− VSMCs treated with or without 8-pCPT-cGMP (data not shown).

Results shown in Fig. 4 also indicated that loss of RGS2 greatly augmented the magnitude of integrated Ca^{2+} responses elicited either in the absence or presence of 8-pCPT-cGMP. In the absence of 8-pCPT-cGMP, AVP-triggered Ca^{2+} responses were 2-fold greater in RGS2−/− cells relative to wild-type controls. In the presence of 8-pCPT-cGMP, AVP-elicited Ca^{2+} responses were 4- to 5-fold greater in RGS2−/− VSMCs relative to wild-type controls. Loss of RGS2 did not affect intracellular Ca^{2+} stores either in the presence or absence of 8-pCPT-cGMP, as indicated by quantifying the integrated Ca^{2+} responses elicited by ionomycin in the absence of extracellular Ca^{2+}. Therefore, A- or MA-VSMCs lacking RGS2 exhibited profoundly defective cGMP-mediated inhibition of AVP-induced Ca^{2+} signaling as well as significantly augmented Ca^{2+} transients with or without treatment with cGMP analogs. RGS2 deficiency thereby may impair vascular relaxation at least in part by blocking the inhibitory action of the NO-cGMP pathway on vasoconstrictor-induced Ca^{2+} signaling.

Expression and Activation of PKG1 in VSMCs Is Independent of RGS2. One possible explanation for the results described above was that PKG1 expression or activation is significantly impaired in VSMCs from RGS2−/− mice. However, results of immunoblotting experiments argued against this hypothesis. The levels of PKG1α and β (the two splice variants expressed in VSMCs) were indistinguishable in wild-type and RGS2−/− A-VSMCs (Fig. 5A; MA-VSMCs could not be obtained in quantities sufficient for immunoblotting experiments). Furthermore, PKG1 activity was stimulated similarly in wild-type and RGS2−/− A-VSMCs after incubation with the specific activator 8-pCPT-cGMP. This conclusion was drawn from the results of immunoblotting experiments that detected similar levels of endogenously expressed VASP phosphorylated on serine-239 (47-kDa band recognized by a monoclonal antibody specific for phosphoserine-239 of VASP), or on serine-239 and serine-157 (50-kDa band recognized by monoclonal antibodies specific for phosphoserine-239 or phosphoserine-157 of VASP; both are established in vivo PKG1 phosphorylation sites; Smolenski et al., 1998; Aizawa et al., 2003) in wild-type and RGS2−/− aortic VSMCs (Fig. 5B). Therefore, VSMCs lacking RGS2 did not

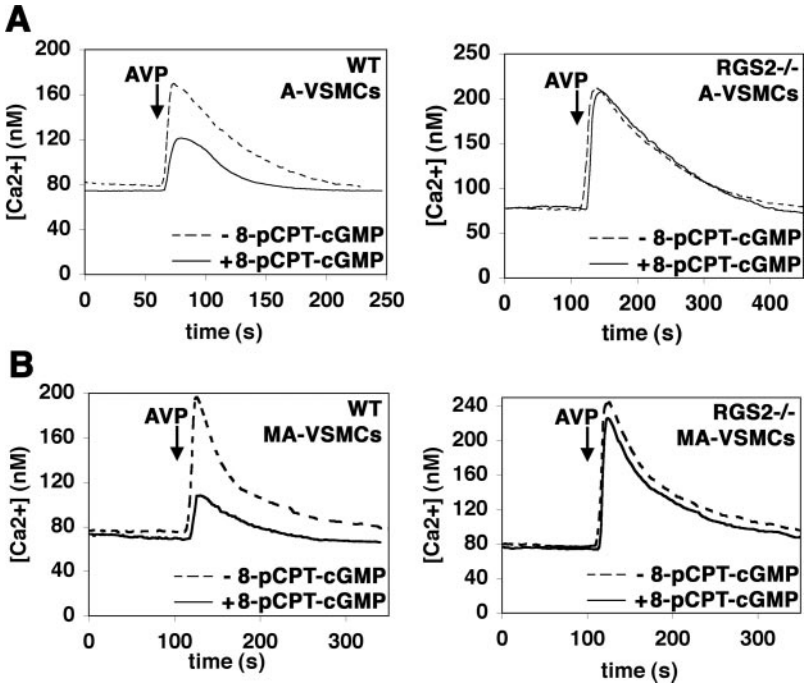

Fig. 3. RGS2 mediates the ability of cGMP analogs to attenuate signaling by vasopressin receptors in vascular smooth muscle cells. Increases $[Ca^{2+}]_i$ elicited by AVP were recorded in Fura-2-loaded primary smooth muscle cells from aorta (A; A-VSMCs) and mesenteric resistance arteries (B; MA-VSMCs) from wild-type and RGS2−/− mice as described in Fig. 2. Cells were treated 10 min without or with 8-pCPT-cGMP (100 μM) before stimulation with AVP (100 nM) at the indicated time. Time courses shown are the average of five independent experiments.

exhibit detectable impairment of PKG1 expression or activation, suggesting that RGS2 functions downstream of PKG1. Therefore, together the results presented above indicated that RGS2 is a novel effector of the NO-cGMP signaling pathway that is required to inhibit vasoconstrictor-induced Ca^{2+} signaling, promote relaxation of the resistance vasculature, and regulate blood pressure.

Discussion

The results of this investigation indicate that RGS2, a negative regulator of signaling by Gq-coupled receptors, is one of several molecules that mediate the action of NO on blood pressure and vasoconstrictor signaling in smooth muscle cells from the resistance vasculature. Therefore, they provide new insight into the mechanisms that regulate blood pressure, vascular tone, and smooth muscle cell signaling, which may be relevant to the genesis or treatment of hypertension, as discussed below.

The NO-cGMP pathway provides a principal means by which blood pressure and vascular tone are regulated because it uses several mechanisms that inhibit the action of vasoconstrictors. Our findings indicate that one of these mechanisms requires RGS2 because the ability of an NO donor to antagonize the pressor effects of phenylephrine is impaired in RGS2−/− mice. RGS2 could mediate the action of the NO-cGMP pathway in several tissues because it is widely expressed, including in vascular smooth muscle and kidney (for review, see Kehrl and Sinnarajah, 2002). However, smooth muscle of the resistance vasculature is likely to be an important locus of RGS2 function because we found that the short-term action of an NO donor on blood pressure is impaired in RGS2−/− mice. Furthermore, the loss of RGS2 in primary smooth muscle cells from mesenteric resistance arteries increases the magnitude and duration of agonist-induced Ca^{2+} responses and blocks the ability of cGMP analogs to attenuate agonist-induced Ca^{2+} signaling. Because expression and activation of PKG1α and β are normal in VSMCs from RGS2−/− mice, our findings strongly support the hypothesis that RGS2 is an essential downstream effector of the NO-cGMP pathway that regulates blood pressure by attenuating vasoconstrictor receptor signaling. RGS2 apparently is a direct effector of PKG1 because it can be phosphorylated and activated by this protein kinase (Tang et al., 2003).

How does RGS2 mediate the inhibitory effects of the NO-cGMP pathway on vasoconstrictor-induced Ca^{2+} signaling? As shown in Fig. 6, we propose that RGS2 attenuates signaling by Gq, the G protein coupled to many vasoconstrictor receptors. This hypothesis is supported by studies indicating that RGS2 is a potent GTPase-activating protein that is selective for Gq (Heximer et al., 1997, 1999; Ingi et al., 1998). Therefore, loss of RGS2 is hypothesized to increase the lifetime of Gqα-GTP, resulting in greater or abnormally prolonged activation of phospholipase Cβ, production of IP_3, and release of Ca^{2+} from intracellular stores or entry of Ca^{2+} from the medium. Indeed, augmented agonist-induced IP_3 production has been shown to occur RGS2−/− cells (Wang et al., 2004). Thus, we propose that the NO-cGMP pathway triggered by the action of vasodilatory agonists inhibits vasoconstrictor-induced Ca^{2+} signaling by activating PKG1, which phosphorylates RGS2, increasing its activity as a GAP for Gqα and promoting its association with the membrane (Tang et al., 2003).

Activation of RGS2 by the NO-cGMP pathway seems to be crucial for inhibiting Ca^{2+} signaling triggered by Gq-coupled vasoconstrictor receptors. Whereas PKG1 activation strongly attenuates (>60%) vasoconstrictor-induced Ca^{2+} responses in wild-type cells, it has little inhibitory effect (~5%) in RGS2 −/− cells. This profound regulatory deficit is remarkable because several other mechanisms allow PKG1 to attenuate Ca^{2+} signaling, including activation of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase via phospholamban phosphorylation and inhibition of IP_3 receptors by IRAG phosphorylation (Raeymaekers et al., 1988; Cornwell et al., 1991; Schlossmann et al., 2000; Ammendola et al., 2001).

However, the profound regulatory deficit in RGS2−/− VSMCs is less surprising when one considers that RGS2 controls an early amplification step (i.e., Gq-mediated PLC

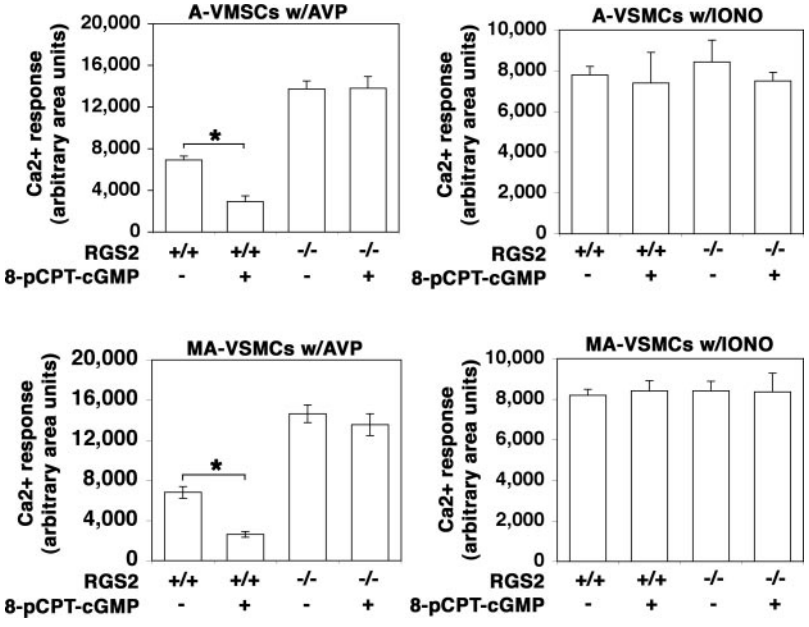

Fig. 4. Quantification of the inhibitory effects of cGMP analogs on vasopressin receptor-mediated Ca^{2+} signaling in vascular smooth muscle cells from wild-type and RGS2−/− mice. Experiments were conducted as described in Fig. 3. The magnitudes of Ca^{2+} responses were quantified by integrating areas under time course curves obtained after stimulating control or 8-pCPT-cGMP-treated cells with AVP (100 nM) or ionomycin (1 μM). Results shown are the average of five independent experiments; S.E.M. is indicated. The inhibitory effect of 8-pCPT-cGMP on AVP-induced Ca^{2+} responses in wild-type cells was statistically significant ($p < 0.001$, indicated by *). The inhibitory effect of 8-pCPT-cGMP on AVP-induced Ca^{2+} responses in RGS2−/− cells was not statistically significant. The inhibitory effect of 8-pCPT-cGMP on intracellular Ca^{2+} pools released by ionomycin in the absence of extracellular Ca^{2+} was not statistically significant.

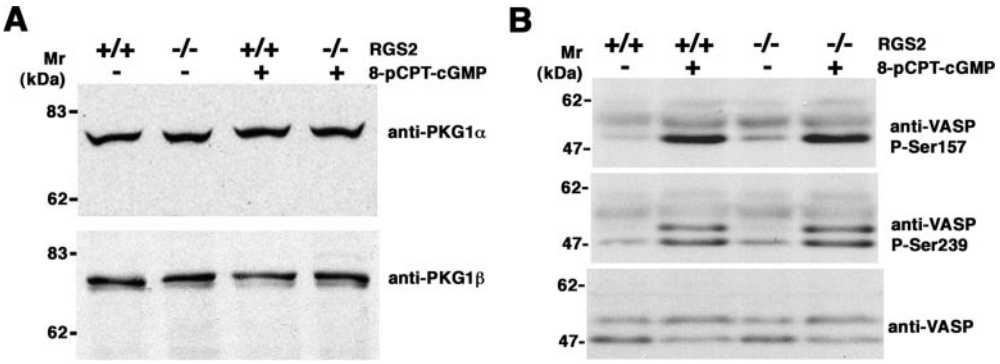

Fig. 5. Expression and activation of PKG1 in vascular smooth muscle cells from wild-type and RGS2−/− mice. Primary aortic vascular smooth muscle cells isolated from wild-type and RGS2−/− mice were treated 30 min with or without 8-pCPT-cGMP (100 μM) and analyzed by immunoblotting with antibodies specific for PKG1α and PKG1β (A) or with antibodies specific for VASP, or VASP specifically phosphorylated on serine-157 or serine-239 (B), which are established PKG phosphorylation sites.

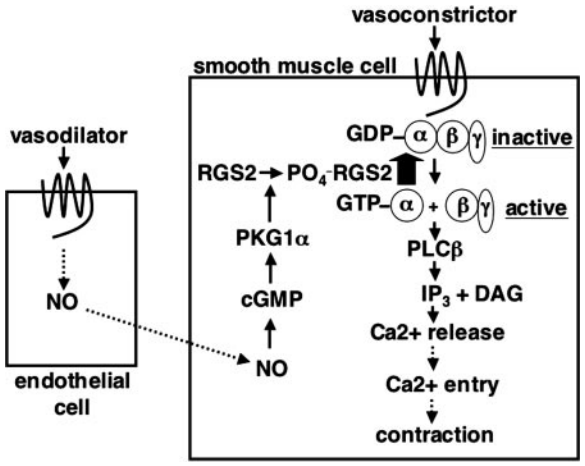

Fig. 6. Model of RGS2 action as an effector of the NO-cGMP pathway that relaxes the vasculature by attenuating vasoconstrictor signaling. Details are presented in the text.

activation and consequent IP_3 and diacylglycerol production) in vasoconstrictor-induced Ca^{2+} signaling pathways. Thus, PKG1-activated regulatory mechanisms controlling Ca^{2+} release and entry either are impaired in cultured RGS2−/− VSMCs or they lack the capacity to compensate adequately for the abnormally strong or prolonged second messenger signals resulting from augmented activation of Gq in cells lacking RGS2.

Although the loss of RGS2 has similar effects on vasoconstrictor-induced Ca^{2+} signaling in VSMCs from aorta and mesenteric resistance arteries, two observations suggest that RGS2 deficiency differentially affects the function of the aorta and resistance vasculature. First, loss of RGS2 seems to augment vasoconstrictor-induced contraction of aortic rings but not the resistance vasculature (Heximer et al., 2003; Tang et al., 2003). Second, RGS2 deficiency impairs NO-mediated relaxation of the resistance vasculature, but not endothelium-intact aortic rings (Tang et al., 2003). Assuming that these differences are not caused by the distinct means by which contraction and relaxation of aortic rings and the resistance vasculature have been analyzed, they suggest that RGS2 regulates contraction and relaxation of the aorta versus resistance arteries in significantly different ways. Whether or how this occurs is not yet clear because the loss of RGS2 has similar effects on vasoconstrictor-induced Ca^{2+} signaling in VSMCs from aorta and mesenteric resistance arteries. However, other signaling pathways, such as those using RhoA or Erk, that regulate contraction and relaxation or endothelium-smooth muscle communication could be affected differentially in aorta versus resistance arteries by RGS2 deficiency.

It is striking that RGS2 deficiency nearly completely impairs the inhibitory action of cGMP analogs on vasoconstrictor-induced Ca^{2+} transients in VSMCs from mesenteric resistance arteries but does not completely block the action of an NO donor on blood pressure. These results support the hypothesis that the NO-cGMP pathway uses several mechanisms to promote vascular relaxation in vivo, some of which do not require RGS2. For example, RGS2 may not be required for cGMP-mediated relaxation caused by inhibition of RhoA action because G_{12}/G_{13} (which are not regulated by RGS2) rather than Gq/11 may provide the primary means by which vasoconstrictor receptors activate RhoA (Gohla et al., 2000).

VSMCs express several closely related RGS proteins, including RGS2, RGS3, and RGS5 (Wang et al., 2002), each of which probably has crucial roles regulating GPCR signaling in the vasculature. These RGS proteins probably are at least partially redundant because each can regulate Gq signaling and the loss of a single RGS protein (RGS2) only modestly prolongs the kinetics of Ca^{2+} signaling. However, RGS2, RGS3, and RGS5 clearly have nonoverlapping functions in smooth muscle cells. Indeed, knockdown studies indicate that RGS3 and RGS5 regulate Gq signaling in a receptor-selective manner (Wang et al., 2002). Furthermore, our results show that RGS2 is required for cGMP-mediated inhibition of vasoconstrictor-triggered Ca^{2+} signaling. In the absence of RGS2, RGS3 and RGS5 may be unable to carry out this function because they lack sites equivalent to those in RGS2 (Ser-46 and Ser-64) that are phosphorylated by PKG1 (Tang et al., 2003). Thus, RGS2 seems to be unique among these proteins in its ability to serve as an effector of the NO-cGMP pathway that attenuates Gq-mediated Ca^{2+} signaling, providing an important and novel cross-talk mechanism for inhibition of vasoconstrictor signaling by vasodilatory agonists.

In conclusion, results presented herein indicate that RGS2 regulates blood pressure to a significant extent by mediating the ability of the NO pathway to relax the resistance vasculature and attenuate vasoconstrictor signaling in vascular smooth muscle cells. Genetic or epigenetic impairment of RGS2 therefore may contribute to the genesis of hypertension. This hypothesis is supported by a recent study showing that RGS2 mRNA expression is down-regulated in the vessel wall in a rat N^{ω}-nitro-L-arginine methyl ester model of hypertension (Dupuis et al., 2004). Therefore, pharmacological agents that up-regulate RGS2 expression or activity may

augment the action of vasodilatory agonists and provide a novel means of treating hypertension.

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Address correspondence to: Dr. Kendall J. Blumer, Department of Cell Biology and Physiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. E-mail: kblumer@cellbio.wustl.edu