Loss-of-Function Mutations in Human Regulator of G Protein Signaling RGS2 Differentially Regulate Pharmacological Reactivity of Resistance Vasculature

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ABSTRACT

Regulator of G protein signaling 2 (RGS2) plays a role in reducing vascular contraction and promoting relaxation due to its GTPase accelerating protein activity toward Gq. Previously, we identified four human loss-of-function (LOF) mutations in RGS2 (Q2L, D40Y, R44H, and R188H). This study aimed to investigate whether those RGS2 LOF mutations disrupt the ability of RGS2 to regulate vascular reactivity. Isolated mesenteric arteries (MAs) from RGS2−/− mice showed an elevated contractile response to 5 nM angiotensin II and a loss of acetylcholine (ACh)–mediated vasodilation. Reintroduction of a wild-type (WT) RGS2-GFP plasmid into RGS2−/− MAs suppressed the vasoconstrictor response to angiotensin II compared with RGS2 response to angiotensin II. RGS2 LOF mutants failed to suppress the angiotensin II constriction response compared with RGS2 WT. In contrast, ACh-mediated vasodilation was restored by expression of RGS2 WT, D40Y, and R44H but not by RGS2 Q2L or R188H. Phosphorylation of RGS2 D40Y and R44H by protein kinase G (PKG) may explain their maintained function to support relaxation in MAs. This is supported by phosphomimetic mutants and suppression of vasorelaxation mediated by RGS2 D40Y or R44H by a PKG inhibitor. These results demonstrate that RGS2 attenuates vasoconstriction in MAs and that RGS2 LOF mutations cannot carry out this effect. Among them, the Q2L and R188H mutants supported less relaxation to ACh, whereas relaxation mediated by the D40Y and R44H mutant proteins was equal to that with WT protein. Phosphorylation of RGS2 by PKG appears to contribute to this vasorelaxation. These results provide insights for precision medicine targeting the rare individuals carrying these RGS2 mutations.

SIGNIFICANCE STATEMENT

Regulator of G protein signaling 2 (RGS2) has been implicated in the control of blood pressure; rare mutations in the RGS2 gene have been identified in large-scale human gene sequencing studies. Four human mutations in RGS2 that cause loss of function (LOF) in cell-based assays were examined in isolated mouse arteries for effects on both vasoconstriction and vasodilation. All mutants showed the expected LOF effects in suppressing vasoconstriction. Surprisingly, the D40Y and R44H mutant RGS2 showed normal control of vasodilation. We propose that this is due to rescue of the mislocalization phenotype of these two mutants by nitric oxide–mediated/protein kinase G–dependent phosphorylation. These mechanisms may guide drug discovery or drug repurposing efforts for hypertension by enhancing RGS2 function.

Introduction

G protein–mediated signaling is critical for the regulation of cardiovascular function, including heart rate, cell growth, and vascular tone. Blockers of G protein–coupled receptor signaling (angiotensin receptor and β and α adrenergic receptor blockers) are among the best therapies for hypertension and heart failure. Regulator of G protein signaling (RGS) proteins accelerate the rate of GTP hydrolysis by active Gaq and Goq subunits, thereby reducing the amplitude and duration of G protein–coupled receptor/G protein signaling. Among more than 20 RGS proteins, RGS2 is highly expressed in vascular smooth muscle cells (VSMCs), where it is regulated by nitric oxide (NO) and controls vascular constriction and relaxation (Tang et al., 2003; Sun et al., 2005).

Biochemically, RGS2 negatively regulates Goq, which transduces signals from a variety of vasoconstrictors. Consistent with a role in vascular control, RGS2 knockout mice are hypertensive (Heximer et al., 2003) and hypertensive human patients had reduced RGS2 mRNA in peripheral

ABBREVIATIONS:  ACh, acetylcholine; AngII, angiotensin II; CHO, Chinese hamster ovary; EDHF, endothelium-derived hyperpolarizing factor; GAP, GTPase accelerating protein; HEK, human embryonic kidney; LOF, loss of function; MA, mesenteric artery; NO, nitric oxide; PB, phosphate buffer; PE, phenylephrine; PKG, protein kinase G; PM, plasma membrane; PSS, physiologic salt solution; RGS, regulator of G protein signaling; SNP, single nucleotide polymorphism; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid; VSMC, vascular smooth muscle cell; WT, wild type.
blood mononuclear cells compared with controls (Semplicini et al., 2006). Furthermore, hypertensive patients were more likely to have the single nucleotide polymorphism (SNP) C1114G in the 3’ untranslated region of the RGS2 gene, which correlated with lower RGS2 protein expression in cultured fibroblasts isolated from skin biopsy (Semplicini et al., 2006). Several rare coding SNPs were found in a Japanese hypertensive cohort at a frequency higher than normotensive controls but the sample size was too small to reach statistical significance (Yang et al., 2005). Subsequent studies confirmed loss of function (LOF) of these SNPs in vitro and defined mechanisms (Bodenstein et al., 2007; Gu et al., 2008). We recently identified four LOF mutations in human RGS2 (Phan et al., 2017) from a set of 16 variants of unknown significance found in the Genome Aggregation Database (Lek et al., 2016). Although they are biochemically characterized, the functional consequences of these mutations in the complex milieu of vascular tissue are unknown. Here, we used an ex vivo method in mesenteric arteries (MAs) to examine how these mutations affect vascular reactivity. We hypothesized that the LOF phenotype of RGS2 mutants identified in a cell-based assay would have LOF physiologic consequence in the MAs.

Materials and Methods

Ethical Approval. All experimental procedures were approved by the Michigan State University Animal Care and Use Committee and were performed in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health.

Animals. C57Bl/6 mice with wild type (WT) and RGS2+/− genotypes were obtained from Dr. Kendall Blumer (Washington University School of Medicine, St. Louis) (Osei-Owusu et al., 2015). Mice were provided access to food and water ad libitum in the Michigan State University animal facility on a 12-hour light/dark cycle. All of the experiments were performed on two L-shaped stainless-steel wires inserted into the lumen and fixed at either end with 3- to 5-month-old male mice. Mice were anesthetized by isoflurane, the MA was removed, and they were euthanized by cervical dislocation.

Materials. Rp-8-pCPT-cGMPS (item 18445, CAS 208445-07-2) was purchased from Cayman Chemical. Angiotensin II (AngII; Sigma-Aldrich) was purchased from Sigma-Aldrich. Angiotensin II was purchased from Cayman Chemical. Angiotensin II (AngII; Sigma-Aldrich) was purchased from Sigma-Aldrich. Arteries were purchased from Sigma-Aldrich (St. Louis, MO).

Pressure Myography. MAs were isolated free of adipose and connective tissue in a physiologic salt solution (PSS) containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl2·6H2O, 10 mM HEPES, and 10 mM dextrose, pH 7.4, at 4°C. Arteries were mounted between two glass holders MyoVIEW II 2.0 (Grass Instruments, Quincy, MA). The basal tension of the aortic ring was maintained at 0.5 g. Changes in isometric force after adding PE in a cumulative manner were recorded on a Grass polygraph (Grass Instruments).

Reversible Permeabilization. Reversible permeabilization of isolated MAs to introduce RGS2 plasmids to the MAs was achieved by a modification of previous methods (Leah et al., 1995; Welsh et al., 2002). Briefly, MAs from RGS2−/− mice were loaded with RGS2-GFP constructs by sequential incubation in the following solutions: 1) 10 mM EGTA, 120 mM NaCl, 5 mM Na2ATP, 2 mM MgCl2, and 20 mM 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminooethanesulfonic acid (TES) (pH 6.8); 20 minutes, 4°C; and 2) 0.1 mM EGTA, 120 mM KCl, 5 mM Na2ATP, 2 mM MgCl2, 20 mM TES, and 1 μg/ml RGS2-GFP plasmids (pH 6.8; overnight, 4°C). Subsequently, arteries were transferred to the following solutions: 3) 0.1 mM EGTA, 120 mM KCl, 5 mM Na2ATP, 10 mM MgCl2, and 20 mM TES (pH 6.8; 30 minutes, 4°C); and 4) 140 mM NaCl, 5 mM KCl, 10 mM MgCl2, 5.6 mM glucose, and 2 mM 3-[N-morpholino]propane-sulfonic acid (pH 7.1, 30 minutes, room temperature). Then [Ca2++] was gradually increased from 0.001 to 0.01 to 1.6–2.4 mM every 10 minutes by adding CaCl2 to solution 4. Arteries were then incubated in Dulbecco’s modified Eagle’s medium/F-12 culture medium (catalog number 11039-021; Gibco, Carlsbad, CA) supplemented with 50 U/ml penicillin and 50 ng/ml streptomycin and maintained at 37°C, 5% CO2 in an incubator. MAs expressing RGS2-GFP were used for myography 24–48 hours after being transferred to culture medium.

Immunofluorescence. After reversible permeabilization, MAs were fixed in 4% paraformaldehyde for 24 hours and rinsed with phosphate buffer (PB), pH 7.2. The arteries were permeabilized in DMSO 100% for 10 minutes, then incubated in blocking buffer/PB buffer containing 4% donkey serum (catalog number D9663, lot SLBQ9773V; Sigma-Aldrich) for 30 minutes, and then incubated with rabbit anti-GFP antibody (catalog number A6455; Life Technologies, Carlsbad, CA) at 1:500 dilution at 4°C overnight. The arteries were then rinsed with 0.2% Triton X-100 in PBS and incubated in Cy3/anti-rabbit secondary antibody (code 711-165-152, Jackson ImmunoResearch, West Grove, PA) at 1:300 dilution at room temperature. The arteries were mounted using ProLong Gold Antifade Mountant with 4’,6-diamidino-2-phenylindole (catalog number P36941; Life Technologies) and visualized using a 40× objective in an Olympus Fluoview 1000 laser scanning confocal microscope.

Cell Culture and Transfection. Cell lines were maintained in a humidified incubator at 37°C with 5% CO2. Human embryonic kidney (HEK)-293 cells and Chinese hamster ovary (CHO)-K1 cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (Gibco) and F-12 nutrient mixture (MTI-GlobalStem, Gaithersburg, MD) were used to transfect HEK-293 cells and CHO-K1 cells according to the manufacturers’ recommended protocols. Transfection mixes were prepared in antibiotic-free Opti-MEM (Gibco). Experiments were run 24–48 hours after transfection.

Confocal Microscopy. HEK-293 cells were seeded onto collagen-coated 35-mm glass-bottom Petri dishes (MatTek Corporation, Ashland, MA) and transfected with RGS2-GFP plasmid constructs along with plasmid DNA encoding Gq12/13 or pDNA3.1 as the control. An Olympus FluoView 1000 laser scanning confocal microscope with a 60× oil immersion objective using 488-nm excitation and 505- to 530-nm emission wavelengths for GFP was used for live cell imaging. This experiment was done 24 hours post-transfection. Representative images of at least 80 live cells per condition from three independent
experiments were acquired. The line scan analysis function in ImageJ (Schneider et al., 2012) was used to perform densitometric quantitation of protein expression.

Ca\(^{2+}\) Mobilization Assay. CHO cells were seeded into black, flat, clear-bottom 384-well plates (Greiner Bio-one, Kremsmünster, Austria) 24 hours post-transfection. Cells were allowed to attach overnight. After the media were removed, cells were loaded with 1× Fluo-4 NW (Molecular Probes, Eugene, OR) in a loading buffer containing Hanks' basal saline solution and 20 mM HEPES, pH 7.4, supplemented with 2.5 μM probenecid. The cell plates were incubated for 30 minutes at 37°C, followed by 30 minutes at room temperature. The Fluo-4 NW was removed and loading buffer was added at 20 μl/well to the cell plate. A range of concentrations of AngII (catalog number A9525; Sigma) at 2× the final concentration was freshly prepared in loading buffer supplemented with 0.1% bovine serum albumin in loading buffer containing 2.5 mM HEPES, pH 7.4. Assay plates were placed in the plate reader and changes in intracellular free calcium in response to the receptor activation were measured.

Statistical Analyses. Data are presented as means ± S.E. Vessel diameter data were analyzed by the t test or two-way ANOVA followed by the indicated multiple-comparisons test. All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA). Statistical significance was denoted by P < 0.05 or as indicated in the figure legends.

**Results**

RGS2 Deficiency Enhances Vascular Contractility to PE and AngII and Causes Impairment of ACh-Mediated Vasorelaxation. Vascular responses of RGS2-deficient arteries to vasoconstrictors and vasodilators have been extensively studied in different vascular beds, including the aorta, MA, renal interlobar, and uterine artery (Tang et al., 2003; Hercule et al., 2007; Osei-Owusu et al., 2012; Jie et al., 2016). Here we assessed the response of MA and aorta to PE, AngII, and ACh with the goal of: 1) validating the effect of RGS2 deficiency on vascular reactivity and 2) optimizing experimental conditions for studies of RGS2 mutant proteins in the context of vascular tissue. Second-order MAs and rings from the thoracic aorta from male RGS2\(^{+/+}\) and RGS2\(^{-/-}\) mice were used to determine how RGS2 protein affects vascular reactivity to different vasoactive substances. PE, which activates the G\(_\alpha_q\)-coupled α1 adrenergic receptor, caused a concentration-dependent contraction in RGS2\(^{+/+}\) MAs and aorta; this response was significantly augmented in RGS2\(^{-/-}\) aortic rings in wire myography (Fig. 1B) but not in pressurized MAs from RGS2\(^{-/-}\) mice (Fig. 1A). The MAs isolated from RGS2\(^{-/-}\) mice did show increased responsiveness to 5 nM AngII (28% ± 1.8% contraction in RGS2\(^{-/-}\) vs. 10% ± 2.6% in RGS2\(^{+/+}\), P < 0.01, t test; Fig. 1C; Supplemental Fig. 1A). In addition, the maximum of ACh-mediated vasodilation in RGS2\(^{-/-}\) MA preconstricted with 5 μM PE was markedly impaired (48.6% ± 8.4% relaxation in RGS2\(^{-/-}\) vs. 92.7% ± 6.2% relaxation in RGS2\(^{+/+}\), P < 0.01 and P < 0.0001, respectively, in two-way ANOVA with the Sidak post-test; Fig. 1D). We chose to focus on testing the reactivity of MAs expressing RGS2 mutants to AngII and ACh in subsequent experiments because MAs contribute to vascular resistance, which determines arterial blood pressure (Christensen and Mulvany, 1993).

Expression of RGS2-GFP in Cultured RGS2\(^{-/-}\) MAs. Using reversible permeabilization to deliver plasmids into intact arteries, we were able to express RGS2-GFP constructs in MAs. Immunofluorescent staining of GFP showed that RGS2-GFP was present in the MA between 48 and 72 hours after reversible permeabilization but absent with a pcDNA3.1 transfection control (Fig. 2A). The staining also showed that intact arteries, we were able to express RGS2-GFP constructs in MAs from RGS2\(^{-/-}\) mice were used to determine how RGS2 deficiency in RGS2\(^{-/-}\) mice showed greater sensitivity to PE than aorta from RGS2\(^{+/+}\) mice. (C) MA response to AngII (5 nM). (D) Concentration response of ACh-induced vasodilation expressed as percentages of the increase in arterial diameter after constriction with PE (3 × 10\(^{-6}\) M) (n = 5). Data are expressed as the mean ± S.E. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 vs. WT (two-way ANOVA with Sidak post-test). n indicates the number of mice used to isolate vessels for each experiment.
z-stack imaging by confocal microscopy. RGS2-GFP was expressed in the VSMC layer and virtually absent RGS2-GFP in the endothelial cell layer (Fig. 2B), based on the depth in the z-stack and morphology of cell nuclei/elongated nuclei in VSMCs and round nuclei in endothelial cells (Luo et al., 2016).

**RGS2 LOF Mutations Fail to Suppress AngII-Induced Vasoconstriction.** To examine the effect of RGS2 mutants in regulating vasoconstriction, we expressed plasmid constructs encoding RGS2 WT and mutants in RGS2−/− MAs by reversible permeabilization versus empty vector as the control. For these experiments, we used a higher concentration of AngII for cultured MAs (50 nM instead of 5 nM in freshly isolated arteries). In pilot studies with transfected RGS2 knockout MAs, 30 nM AngII gave only a small contractile response, whereas 50 nM gave a response similar to that elicited by 5 and 50 nM in untransfected MAs (Supplemental Fig. 1B). The 50 nM AngII provided sufficient significant vasoconstriction to be able to observe effects of RGS2 in transfected arteries. This is potentially due to impaired receptor function after reversible permeabilization. MAs expressing empty vector or RGS2−/− MAs without RGS2 transfection showed 34.3% ± 4.7% (Fig. 3A) and 30.0% ± 3.1% (Fig. 3B) contraction relative to baseline, respectively. Expression of RGS2 WT was able to suppress contraction in two independent experiments. In MAs expressing RGS2 WT, AngII caused a reduction in arterial diameter of only 4.2% ± 1.3% (P < 0.01, one-way ANOVA, Dunnett post-test; Fig. 3A) and 7.2% ± 2.6% (P < 0.05, one-way ANOVA, Dunnett post-test; Fig. 3B). Two mutants identified in our previous work as having normal function, RGS2 M5V and A99G (Phan et al., 2017), resulted in a similar level of vascular contraction to RGS2 WT (6.8% ± 3.6% and 10.9% ± 4.2%, respectively; Fig. 3B). The RGS2 Q2L mutant gave reduced suppression of contraction compared with RGS WT (18.8% ± 6.6% constriction vs. 4.2% ± 1.3%); however, the difference was not statistically significant (P = 0.09) (Fig. 3A). Arteries expressing RGS2 D40Y, R44H, and R188H mutants showed a significantly elevated contractile response to 50 nM AngII stimulation (28.3% ± 7.7%, 25.7% ± 8.7%, and 29.8% ± 5.5%, respectively) compared with arteries expressing RGS2 WT (4.2% ± 1.3%, P < 0.05, one-way ANOVA, Dunnett post-test) (Fig. 3).

**RGS2 LOF Mutations Differentially Affect ACh-Evoked Vasodilation.** Because freshly isolated RGS2−/− MAs show reduced relaxation to ACh, LOF mutations of RGS2 may behave similarly. To probe the function of these mutants in mediating ACh-induced vascular relaxation, we measured ACh-evoked vasodilation of MAs expressing WT and mutant RGS2. Using MAs preconstricted with PE (3 μM), we found that cultured RGS2-deficient MA (transfected with empty vector) showed a maximal relaxation of 52.8% ± 14.3% (Fig. 4). This is similar to the response of freshly isolated RGS2−/− MAs (48.6% ± 8.4%; Fig. 1C) and much less than that of arteries expressing WT RGS2 (99.6% ± 5.5%, P = 0.0001, two-way ANOVA with Dunnett post-test; Fig. 4). Among the four LOF mutants, we found that ACh-mediated relaxation with the RGS2 Q2L and R188H mutants was significantly impaired compared with RGS2 WT (Q2L: 75% ± 7%, P < 0.05; R188H: 63.8% ± 14.3%, P < 0.001, Dunnett post-test; Fig. 4A). Arteries expressing the D40Y and R44H mutant RGS2, however, showed an equivalent relaxation response to those with RGS2 WT (D40Y: 98.4% ± 12.3%; R44H: 93.98% ± 4.8%; Figs. 4B and 5E). It is notable that these latter two mutations reduce RGS2 function by perturbing membrane localization, whereas the other two have different mechanisms (Phan et al., 2017).
Ser46 and Ser64 Phosphomimetic Mutants of D40Y and R44H RGS2 can Rescue Protein Plasma Membrane Localization and Function. In VSMCs, RGS2 has been shown to be an effector of the NO-cGMP pathway. RGS2 is phosphorylated by protein kinase G1 (PKG1a), which is activated by cGMP; as a consequence, RGS2 membrane localization and GTPase accelerating protein (GAP) function is enhanced (Sun et al., 2005; Osei-Owusu et al., 2007). RGS2 phosphorylation at Ser46 and Ser64 by PKG1a increased plasma membrane (PM) localization and GAP activity of RGS2 protein (Tang et al., 2003; Osei-Owusu et al., 2007). Given that the two RGS2 mutants mentioned above (D40Y, R44H) had impaired PM localization in HEK-293 cells but supported a normal ACh-evoked relaxation response in the MAs, we hypothesized that phosphorylation of D40Y and R44H mutant proteins in VSMCs by NO-cGMP-PKG pathway activation upon ACh stimulation is able to rescue the impaired membrane localization and function of these mutants. The effects of site-specific phosphorylation on protein activity can be mimicked by mutation of Ser (S) or Thr (T) to Asp (D). Therefore, to model the effects of phosphorylation of Ser46 and Ser64 on RGS2 protein localization, we performed mutagenesis to make S46D and S64D mutations of tagged constructs encoding WT, D40Y, and R44H (RGS2 WT DD, D40Y DD, and R44H DD). The expression levels of the phosphomimetic mutants were similar to their nonphosphorylated proteins and similar to WT RGS2 (Supplemental Fig. 2). When coexpressed with Gq Q209L, D40Y, and R44H, mutant RGS2 remained in the nucleus, whereas RGS2 WT and the phosphomimetic mutants (RGS2 DD, D40Y DD, and R44H DD) localized to the PM (Fig. 5, A and B). In addition, the RGS2 D40Y and R44H mutants supported less Ca2+ inhibition compared with RGS2 WT (Fig. 5C), whereas RGS2 D40Y DD and R44H DD suppressed AngII receptor type 1–mediated Ca2+ mobilization equivalently to RGS2 DD (Fig. 5D).

Effect of PKG Inhibition on ACh-Mediated Relaxation in MAs Expressing the D40Y RGS2 Mutant. If phosphorylation helps maintain the function of the D40Y mutant RGS2, then inhibition of PKG with Rp-8-pCPT-cGMPS (30 μM) should prevent the rescue of relaxation.

Fig. 3. LOF mutations of RGS2 failed to suppress AngII-mediated vasoconstriction. RGS2-GFP constructs were introduced to MAs isolated from RGS2−/− mice by reversible permeabilization. (A) RGS2 WT- and M5V- and A99G-expressing (two normal function mutations; Phan et al., 2017) arteries reduced vascular contraction upon 50 nM AngII stimulation, whereas the other mutants did not. (B) D40Y-, R44H-, and R188H mutant-expressing arteries showed a significantly elevated contractile response to 50 nM AngII stimulation compared with RGS2 WT-expressing arteries. *P < 0.05 compared with empty vector; **P < 0.05 compared with WT (one-way ANOVA with Dunnett post-test).

Fig. 4. Differential effects of RGS2 missense mutations on ACh-mediated vasorelaxation. (A) RGS2-GFP expression in RGS2−/− MAs rescued ACh-mediated relaxation. RGS2 WT-expressing arteries showed enhanced relaxation compared with empty vector transfected arteries. Q2L- and R188H-expressing arteries showed less relaxation compared with RGS2 WT-expressing arteries. *P < 0.05 compared with empty vector test.)
responses of MAs. PKG inhibition slightly reduced maximal relaxation of MAs expressing WT RGS2 from 99.6% ± 5.5% to 87.8% ± 4.2% (not significant) and shifted the ACh concentration-response curve a half-log to the right (logEC50 5.80 ± 0.06 vs. 6.30 ± 0.09, not significant; Fig. 5, E and F; Table 1). The relaxation response of RGS2−/− MAs transfected with empty vector was impaired (maximum relaxation 52.9% ± 14.3%) and was further depressed by PKG inhibition (maximum relaxation 38.4% ± 3.5%, not significant; Fig. 5, E and F; Table 1). However, PKG inhibition drastically reduced the dilation of MAs transfected with the D40Y mutant RGS2 (40.3% ± 6.7% vs. 98.4% ± 12.3%, P < 0.001; Fig. 5, E and F;
In addition, we show for the first time that MAs from RGS2 ACh in WT and RGS2-deficient MAs (Osei-Owusu et al., 2012). We were able to confirm the previously published effects of localization in HEK-293 cells and restore the vasorelaxation appears that PKG-dependent phosphorylation can rescue PM attributed to phosphorylation of RGS2 by PKG in VSMCs. It that all four mutant proteins failed to effectively suppress AngII-mediated vasoconstriction in mouse MAs. RGS2 WT and D40Y to ACh demonstrated (Osei-Owusu et al., 2012) with our method. However, overexpression of RGS2 in VSMC appears to be important in mediating ACh-evoked relaxation, since overexpression of the WT RGS2 in VSMCs was able to rescue the impaired ACh-mediated relaxation in RGS2/−/− arteries. In addition, mutants with different LOF mechanisms (Phan et al., 2017) behaved differently (Fig. 4).

ACh mediates relaxation in part by activating the NO-cGMP-PKG pathway in endothelium-intact arteries (Christopoulos and El-Fakahany, 1999; Sun et al., 2005). RGS2, as an effector of PKG, has previously been shown to be phosphorylated at Ser46 and Ser64 (Tang et al., 2003), enhancing PM localization. These phosphosites, interestingly, are located close to the sites of the D40Y and R44H mutations in RGS2, as shown in a model of the RGS2 N-terminal helix (Fig. 6) interacting with lipids (Tikhonova et al., 2006). This helix facilitates PM targeting (Heximer et al., 2001; Gu et al., 2007). Introducing phosphomimetic mutants at two PKGα phosphorylation sites of RGS2 (D40Y DD, R44H DD) in the D40Y and R44H mutant RGS2 proteins enhanced PM localization and function in suppressing Ca2+ release (Fig. 5, A, B, and D). Conversely, PKG inhibition substantially reduced ACh-induced relaxation in RGS2 D40Y expressing RGS2/−/− MAs (Fig. 5F). These results support our hypothesis that the normal ACh-mediated relaxation response of the D40Y and R44H mutant RGS2 in MAs is due to phosphorylation of mutant proteins by PKG. At the molecular level, this phosphorylation may rescue PM localization of the mutant proteins. The RGS2 Q2L and R188H mutants may not be rescued by phosphorylation since the mechanism of their LOF behavior is not related to PM localization.

Although the RGS2 D40Y and R44H mutant alleles supported a normal relaxation response in resistance arteries, an intact endothelial NO-cGMP pathway was required for this response. When endothelial function is compromised such as in hypertension (Brandes, 2014) or in conditions wherein PKG function is deficient (Michael et al., 2008), RGS2 proteins expressed from these mutant alleles will likely be unphosphorylated, triggering aberrant Gq signaling and an impaired relaxation response.

**How Does Phosphorylation of RGS2 at Ser46 and Ser64 Rescue D40Y and R44H Membrane Localization?** The RGS2-lipid bilayer interaction was impaired in the R44H mutant according to the snorkeling-dependent stabilization model (Gu et al., 2008). In the case of the D40Y mutant, the change from a negatively charged Asp residue to a hydrophobic Tyr residue may disrupt the local environment for the anchorage of the adjacent Trp41 to the PM (Gu et al., 2008). Several mechanisms have been proposed to explain how phosphorylation at these sites might enhance recruitment of RGS2 to the PM. Salt bridge formation between the phosphorylated Ser residues and adjacent Lys residues (Osei-Owusu et al., 2007) may stabilize the N-terminal amphipathic helix. This could explain how introduction of the Asp phosphomimetic in place of Ser at positions 46 and 64 could stabilize the amphipathic α helix disrupted by the D40Y and R44H mutations to rescue function.

RGS2 plays three roles in the vasculature: 1) it functions directly as a GAP to negate vasoconstrictor actions through Gαq, 2) it acts as an effector of NO-cGMP-PKG signaling to promote vasodilation by modulating Gαq-mediated vasoconstrictor action, and 3) it promotes EDHF-mediated vasorelaxation.
(Osei-Owusu et al., 2012). By overexpressing RGS2 in the MAs, we were able to probe the first two functions of these RGS2 mutants in regulating vascular reactivity of MAs. A limitation of this study is the lack of knock-in animal models to allow investigation of whether endogenous RGS2 LOF mutants affect whole-body blood pressure responses. In addition, we are unable to assess their effects on the central nervous system or the kidney. Nevertheless, our data provide a new understanding how human RGS2 mutants behave in VSMCs in the resistance vasculature. We also provide molecular evidence for functional differences among mutant alleles where the function of D40Y or R44H mutants can be rescued by PKG-mediated phosphorylation. These results provide critical insights for precision therapeutic approaches to individuals bearing particular RGS2 mutations and may facilitate drug discovery to target these rare individuals.

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Authorship Contributions

Participated in research design: Phan, Neubig.
Conducted experiments: Phan.
Contributed new reagents or analytic tools: Jackson, Watts.
Performed data analysis: Phan, Neubig.
Wrote or contributed to the writing of the manuscript: Phan, Jackson, Shaw, Watts, Neubig.

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