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p63RhoGEF Couples $G_{\alpha q/11}$-mediated Signaling to Ca^{2+} Sensitization of Vascular Smooth Muscle Contractility

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Abstract

Rationale—In normal and diseased vascular smooth muscle (SM), the RhoA pathway, which is activated by multiple agonists through G protein-coupled receptors (GPCRs), plays a central role in regulating basal tone and peripheral resistance. This occurs through inhibition of myosin light chain phosphatase, leading to increased phosphorylation of the myosin regulatory light chain. While it is thought that specific agonists and GPCRs may couple to distinct RhoA guanine nucleotide exchange factors (GEFs), thus raising the possibility of selective targeting of specific GEFs for therapeutic use, this notion is largely unexplored for SM contraction.

Objective—We examine whether p63RhoGEF, known to couple specifically to $G\alpha_{q/11}$ *in vitro*, is functional in blood vessels as a mediator of RhoA activation, and if it is selectively activated by $G\alpha_{q/11}$ coupled agonists.

Methods and Results—We find that p63RhoGEF is present across SM tissues and demonstrate that silencing of the endogenous p63RhoGEF in mouse portal vein inhibits contractile force induced by endothelin-1 to a greater extent than the predominantly $G\alpha_{12/13}$ mediated thromboxane analogue, U46619. This is because endothelin-1 acts on $G\alpha_{q/11}$ as well as $G\alpha_{12/13}$. Introduction of the exogenous isolated pleckstrin-homology (PH) domain of p63RhoGEF (residues 331–580) into permeabilized rabbit portal vein inhibited Ca^{2+} sensitized force and activation of RhoA, when phenylephrine was used as an agonist. This reinforces the results based on endothelin-1, because phenylephrine is thought to act exclusively through $G\alpha_{q/11}$.

Conclusion—We demonstrate that p63RhoGEF selectively couples $G\alpha_{q/11}$, but not $G\alpha_{12/13}$, to RhoA activation in blood vessels and cultured cells, and thus mediates the physiologically important Ca^{2+} sensitization of force induced with $G\alpha_{q/11}$ coupled agonists. Our results suggest that signaling through p63RhoGEF provides a novel mechanism for selective regulation of blood pressure.

Keywords

Ca^{2+} sensitization; RhoA; Signal transduction; Vascular smooth muscle; RhoGEF

Introduction

The contractile response in smooth muscle (SM) to agonist stimulation is mediated through the phosphorylation of the myosin regulatory light chain (RLC_{20}). The level of this

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Disclosures
None.

phosphorylation reflects the balance of the opposing activities of the calcium-calmodulin-dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase [1–3]. Although an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]$) serves as a primary on-switch for the activation of MLCK, both MLCK and myosin light chain phosphatase are additionally regulated by diverse kinases, which in turn are regulated by diverse physiological agonists [3, 4]. Importantly, the activity of myosin light chain phosphatase is negatively regulated when Thr853, located in its regulatory, myosin targeting phosphatase subunit (MYPT1) is phosphorylated by the Rho-kinase (ROCK) [1–3]. This inhibition of myosin light chain phosphatase activity results in an increase in force generating phosphorylated myosin motors, without further increase in intracellular $[Ca^{2+}]$. This physiologically important phenomenon is known as Ca^{2+} sensitization [3, 5–7].

The Ca^{2+} sensitization pathway is initiated when ROCK is activated by the GTP-bound, biologically active form of the cytosolic GTPase - RhoA [8]. Like other GTPases of the ***R***as-***ho***mology (Rho) family [9, 10], RhoA functions as a molecular switch; it is biologically inert in the GDP-bound form, but upon exchange of GDP to GTP, it undergoes a conformational change enabling it to interact with various target effectors [11–13]. This results, for example, in the activation of kinases, such as ROCK. RhoA can serve as a switch, because it is a poor hydrolase, with k_{cat} of ~1/min [14], and can therefore exist for considerable time period as a substrate-enzyme complex (the on-state). To switch between the on- and off-states, RhoA is therefore assisted by other regulatory proteins in its catalytic cycle. The GTPase-activating proteins enhance the hydrolysis of GTP to GDP [15, 16], thereby terminating the biological signal. The GDP-bound RhoA is then sequestered in the cytosolic pool by the Rho-nucleotide dissociation inhibitor, RhoGDI [17–19]. Finally, a family of guanine nucleotide exchange factors (GEFs) catalyze the loading of GTP for GDP, re-initiating the signaling cycle [20]. Both GTPase-activating proteins and GEFs are themselves tightly regulated upstream of RhoA.

Basal tone and the contractile state of SM are regulated by multiple agonists acting through G-protein coupled-receptors (GPCRs) leading to a rise in intracellular $[Ca^{2+}]$. However, the magnitude of force at a specific level of $[Ca^{2+}]$ is highly variable depending on the nature of the stimulus and on the vascular bed. In cases where the force/$[Ca^{2+}]$ ratio is high, the force is enhanced due to the activation of a RhoA/ROCK signaling pathway, and consequently Ca^{2+} sensitization. Thus, diverse agonists acting on heterotrimeric GPCRs can exert different magnitudes of RhoA-mediated $[Ca^{2+}]$-sensitized contractile force [3, 21–23]. This strongly suggests the presence of coupling in SM between agonist-activated GPCRs and RhoA, most likely through different GEFs. However, it is not clear how many RhoA-specific GEFs are functional in SM, whether specific GEFs are coupled to specific agonists and GPCRs, or if they regulate different functions in SM cells.

Consistent with the notion that specific agonists and GPCRs couple to specific RhoGEFs, a small family of GEFs was recently found to be activated directly by $G\alpha_{12/13}$-coupled receptors [24, 25]. These proteins contain a unique RGSL (***r***egulator of ***G***-protein ***s***ignaling-***l***ike) domain, which recognizes and binds to $G\alpha_{12/13}$ [26–28]. It has been demonstrated that one of these GEFs, the leukemia-associated RhoGEF (LARG) [29] plays a central role in the regulation of vascular SM tone in salt-dependent hypertension, but not in the maintenance of basal blood pressure [30]. The other two related RGSL-containing GEFs, i.e. PDZRhoGEF protein [31, 32] and p115-RhoGEF [33], were also found in SM tissues but whether they couple to specific or multiple agonists and GPCRs is unknown.

Agonist-coupling through GPCRs to $G\alpha_{q/11}$ is thought to be involved in the stimulation of phospholipase C (PLC)-mediated, Ca^{2+}-calmodulin activation of MLCK [3, 22, 34, 35] and activation of RhoA [35, 36]. It has been shown that the RhoA specific GEF, p63RhoGEF [37, 38],

is involved in SM signaling in response to stimulation by angiotensin II [35]. In this paper, we present evidence for the involvement of p63RhoGEF specifically in mediation of $G\alpha_{q/11}$-coupled, agonist-induced Ca^{2+}-sensitized force in SM. We determined that p63RhoGEF mRNA and protein is ubiquitously present in SM cells and in vascular and gastrointestinal tissue across several species, including the human. We further show that following stimulation through $G\alpha_{q/11}$ with agonists such as endothelin-1 (ET-1) and phenylephrine, p63RhoGEF catalyzes GTP exchange on RhoA in SM and regulates Ca^{2+} sensitization.

Methods

All procedures using animals were carried out according to protocols approved by the Animal Care and Use Committee at the University of Virginia. Detailed experimental procedures are in Supplemental Material available at http://circres.ahajournals.org.

Anti-p63RhoGEF antibodies

Two anti-p63RhoGEF antibodies are purchased from Proteintech, Illinois (51004-1-AP and 14839-1-AP).

Expression plasmids and recombinant protein production

Human p63RhoGEF cDNA was PCR-amplified and introduced into pGST-Parallel1 [39], p3xFLAG-Myc-CMV-24 (SIGMA) and pcDNA3 (Invitrogen) plasmids. Glutathione S-transferase (GST)-fused $p63RhoGEF^{331–580}$ recombinant protein was produced in *Escherichia coli* BL21 and purified using glutathione beads. GST was subsequently cleaved off using recombinant tobacco etch virus (TEV) protease.

Smooth muscle contraction experiment

Force measurements on intact, α-toxin or β-escin permeabilized muscles were carried out as detailed in Supplemental Material.

Rhotekin assay

Rabbit portal vein strips were prepared and treated using the same protocols as in the contraction experiments and harvested at each critical time point. Mouse embryonic fibroblast (MEF) cells were transfected with mammalian expression plasmids to over-express FLAG- $p63RhoGEF^{331–580}$. RhoA activity was assayed as detailed in Supplemental Material.

RLC_{20} and MYPT1 phosphorylation

Rabbit portal vein strips were treated using the same protocols as in the contraction assays and processed as described previously [40]. Phosphorylation measurements are detailed in Supplemental Material.

Co-immunoprecipitation assays

Co-immunoprecipitation assays on human embryonic kidney (HEK) 293 cell transfectants (expressing combinations of FLAG-p63RhoGEF-Full-Myc and/or FLAG-$p63RhoGEF^{331–580}$ and/or $G_{\alpha 11}$ wild-type or $G_{\alpha 11}$ Q209T constitutively active mutant) are detailed in Supplemental Material.

p63RhoGEF knock-down

An RNA interference sequence [GCCAAGCTGGATGAAGATGAG] was designed to target both mouse and human p63RhoGEF mRNAs that coincidentally match rat

p63RhoGEF mRNA sequence. Short hairpin RNA (shRNA) was delivered and expressed either by pENTR/U6 plasmid (Invitrogen) or adenovirus including the sequence for the expression of shRNA in mammalian cells.

Quantitative polymerase chain reaction

Total mRNA libraries prepared from unpassaged aortic, pulmonary artery and brain vascular SM primary human cell cultures were purchased from ScienCell Research Laboratories (Carlsbad, California). RNA was also prepared from animal tissue samples. mRNA expression levels of p63RhoGEF and other GEFs were quantitated by RT-PCR.

Statistical Analysis

All data are presented as mean +/− SEM. Differences were considered significant at a P value < 0.05 using 2-tailed Student's t-test.

Results

p63RhoGEF transcription and expression in SM

We chose the mouse as our principal model system. To quantify the level of p63RhoGEF transcription in comparison to those of other GEFs in mouse SM, we performed quantitative RT-PCR using mouse vascular SM tissues. To assess if the transcription patterns are representative of those observed in human, we also screened mRNA libraries from human aorta, pulmonary artery and brain vascular primary, unpassaged SM cells. The p63RhoGEF mRNA was detected in all of the mouse tissues screened and showed particularly high transcription levels in portal vein (Figure 1)— which was subsequently used in our functional assays — as well as in aorta and pulmonary artery. Of significance is the presence of p63RhoGEF mRNA in mouse resistance arteries, such as the thoracodorsal and mesenteric arteries. In human cells, p63RhoGEF mRNA level was the highest in the aorta, followed by pulmonary artery and brain vascular SM cells (Figure 1 inset).

To assess the expression levels of p63RhoGEF we turned to rat tissues, because of the larger body size of the animal. As shown in Figure 2, p63RhoGEF was detected in diverse tissues, except for brain, liver, diaphragm and heart. Similar results were obtained for select mouse and rabbit tissues indicating a consistent trend across species (data not shown). Importantly, we also screened rat tissues for the expression of $G\alpha_{q/11}$, and we discovered that it follows a trend similar to p63RhoGEF. Similarly, RhoA expression was high in SM (Figure 2). Expression of p63RhoGEF was also detected in cultured rat aortic SM cells (R518) and mouse embryonic fibroblast (MEF) cells (Figure 2) but not in human embryonic kidney (HEK) 293 cells (Online Figure I, B). The anti-p63RhoGEF antibody typically gave triplet bands across species by Western blot and the lowest molecular weight band is predominant in mouse samples and is demonstrated to be non-specific (Online Figure I, B). The top band (80 kDa) corresponds to the full-length p63RhoGEF consisting of 580 amino acid residues. Further details of experiments characterizing the p63RhoGEF antibodies and showing that the lowest band is non-specific while the middle band is likely a truncated form of p63RhoGEF are described in the Supplemental Material. While the presence of p63RhoGEF mRNA in brain and heart [38, 41] and HEK293 cells [37] has been reported previously, we only observed the lowest protein molecular weight band in brain samples (Figure 2).

Suppression of mouse portal vein contractility by p63RhoGEF knock-down

Next, we asked if knock-down of p63RhoGEF might suppress contractility in intact blood vessels. Using LacZ-expressing adenovirus, we found that we can successfully infect mouse portal veins and cerebral vessels, but not the aorta or mesenteric arteries. Therefore, we treated mouse portal veins with a viral construct designed to express short hairpin RNA

(shRNA) targeting p63RhoGEF mRNA. This construct was effective as it knocked down the protein expression of p63RhoGEF by up to 79% in cultured cells (Online Figure I, A). In portal vein, after 5 days of treatment with the viral particle, the p63RhoGEF mRNA was reduced by 40%, based on quantitative RT-PCR analysis (Figure 3A). Reduction of p63RhoGEF at the protein level by ~35% was confirmed by Western blot analysis using two pooled mouse portal veins (Figure 3A). Similar effects were also observed in three other Western blot analyses using the same portal vein SM strips used in contraction experiments, although the small sample size did not allow accurate quantification (data not shown).

To probe the impact of the p63RhoGEF knock-down on $G\alpha_{q/11}$ and $G\alpha_{12/13}$-mediated SM contractility, we used, respectively, endothelin-1 (ET-1) and a thromboxane analogue U46619, as agonists. The ET-1 receptor is coupled to $G\alpha_{q/11}$, while U46619 acts primarily through $G\alpha_{12/13}$. Both agonists induce strong contractile response in intact SM through a combination of Ca^{2+} influx, Ca^{2+}-dependent activation of PLC, Ca^{2+} release from intracellular compartments, as well as Ca^{2+} sensitization [42, 43]. Contraction in response to increasing concentrations of ET-1 was significantly lower in p63RhoGEF shRNA-treated vessel strips compared to control (non-targeting) treated vessels (Figure 3B, Online Figure II). Contraction induced by U46619 in shRNA-treated vessels was only slightly lower at the higher concentrations of U46619, than that observed in control treated vessels, although the difference did not reach statistical significance (Figure 3C, Online Figure II).

p63RhoGEF knock-down disrupts the $G\alpha_{q/11}$-coupled Ca^{2+} sensitizationin mouse portal vein

Having established a link between p63RhoGEF and SM contractility in response to ET-1, we set out to examine the effect of reduced p63RhoGEF levels specifically on the RhoA-mediated, Ca^{2+} sensitization pathway. To assess contractility in the absence of Ca^{2+} influx, Ca^{2+} release from intracellular compartments, and other [Ca^{2+}]-dependent phenomena, we studied contractile activity of mouse portal vein permeabilized to ions by α-toxin at a constant buffered intracellular [Ca^{2+}], a protocol that retains receptor-coupling to RhoA [3, 6]. Contraction induced by stimulation with ET-1 at pCa 6.3 was significantly reduced in vessels in which p63RhoGEF expression was reduced, when compared to non-targeting control treated vessels (Figure 4A–C). Subsequent addition of GTPγS (10 μM), as a means to activate RhoA directly bypassing GEF function, resulted in significantly increased contractile responses in vessels where p63RhoGEF expression was reduced (Figure 4A–B). This is consistent with lower levels of activated, GTP-bound RhoA in the knocked-down vessels. In contrast, the U46619 contractile responses of non-targeting and p63RhoGEF shRNA-treated vessels did not differ significantly. The maximal contraction at pCa 4.5, expressed as absolute force, was not affected by knock-down of p63RhoGEF, as evidenced by identical maximal force from identical sizes of vessel strips of the two samples (0.10 ± 0.03 vs 0.10 ± 0.02 mN). Importantly, phosphorylation of MYPT1 at the ROCK target, Thr853 after stimulation with ET-1 was significantly reduced in the p63RhoGEF shRNA-treated portal vein, as compared to controls (Figure 4D), consistent with a reduction in RhoA activity. In contrast, MYPT1 Thr853 phosphorylation was not significantly changed in vessels stimulated with U46619 (Figure 4D).

Ca^{2+} sensitized force is relaxed by the recombinant extended PH domain fragment of p63RhoGEF

It has been established, that in its nascent form, p63RhoGEF is autoinhibited. The molecule contains a Dbl-homology (DH domain), a minimal unit to catalyze the GTP exchange reaction [20, 44], and an adjacent C-terminal pleckstrin-homology (PH) domain, which exerts an autoinhibitory function through an interaction with the DH domain. This mechanism appears to be conserved in the related Dbl family members, Trio and Kalirin [45]. Activation

occurs when the $G\alpha_{q/11}$ subunit binds to specific amino acids in the PH domain plus its adjacent conserved amino acid stretch (extended PH domain) of p63RhoGEF, thus causing a structural reorganization in p63RhoGEF and relief of autoinhibition. We hypothesized that if the Ca^{2+}-sensitization effect is mediated by the same mechanism, then the isolated, recombinant extended PH domain ($p63RhoGEF^{331–580}$) should interfere with the process, either by binding to the activated $G\alpha_{q/11}$ and blocking downstream signaling, or by binding to the transiently open, i.e. active conformation of endogenous p63RhoGEF. To test this hypothesis in intact blood vessels, we needed a vessel that responds to phenylephrine and can be well permeabilized to proteins to allow for the introduction of the p63RhoGEF fragment into the SM cells, while retaining the receptor-coupling to RhoA. Because this was not possible using mouse portal vein, we used instead rabbit portal vein permeabilized with β-escin[46]. Fortuitously, unlike the mouse vessel, rabbit portal vein responds to phenylephrine, reported to act purely through $G\alpha_{q/11}$, and not $G\alpha_{12/13}$ [30], with Ca^{2+} sensitized force at constant intracellular [Ca^{2+}] (pCa 6.3). Phenylephrine/$G\alpha_{q/11}$ coupling was established in mouse aorta[30]. Phenylephrine is a specificα1-adrenoreceptor agonist [47] shown to specifically mediate contractile responses through activation of $G\alpha_{q/11}$ [48]. α1-adrenoreceptors have been shown to be present and functional in rabbit arteries including the carotid aortic, iliac, mesenteric, renal, ear and resistance arteries [47]. Therefore, we assume that they are also present in rabbit portal vein, additionally this vessel contracts in response to phenylephrine. When the contractile force induced by phenylephrine reached a plateau, a control filtrate (i.e. buffer from which recombinant $p63RhoGEF^{331–580}$ protein was removed) was added, followed by the $p63RhoGEF^{331–580}$ protein. The filtrate produced no effect (Figure 5A), while the recombinant $p63RhoGEF^{331–580}$ fragment induced relaxation of the SM strips (Figure 5A; Online Figure III). Both RhoA activity and RLC_{20} phosphorylation level increased with phenylephrine-induced Ca^{2+} sensitized force and both decreased upon introduction of $p63RhoGEF^{331–580}$ (Figure 5B). These findings in blood vessels confirm the hypothesis that recombinant $p63RhoGEF^{331–580}$ inhibits endogenous p63RhoGEF or interferes with the regulatory pathway by sequestering activated $G\alpha_{q/11}$ as predicted from *in vitro* experiments [45].

RhoA-GDP turnover is suppressed in MEF cells by over-expression of $p63RhoGEF^{331–580}$ fragment

Having established an inhibitory role for $p63RhoGEF^{331–580}$ in blood vessels, we investigated if it acts in the same way in cells. We could not use SM cells for that purpose, because their transfection efficiency is generally only < 2–3% by traditional methods and about 30 % by electroporation. Instead, we resorted to mouse embryonic fibroblasts (MEF) where we were able to achieve 80% transfection efficiency. Using MEF cells we investigated the changes in relative levels of RhoA•GTP upon phenylephrine stimulation in the presence and absence of exogenous $p63RhoGEF^{331–580}$. RhoA•GTP levels were increased when MEF cells were stimulated by phenylephrine (5 μM) (Figure 6A), and dropped in the presence of over-expressed $p63RhoGEF^{331–580}$. A similar trend was also observed upon ET-1 stimulation (data not shown).

$p63RhoGEF^{331–580}$ associates with full-length p63RhoGEF and the constitutively active $G\alpha_{q/11}^{Q209L}$

We then asked if $p63RhoGEF^{331–580}$ interferes with the RhoA activation pathway by interacting with $G\alpha_{q/11}$ or with endogenous p63RhoGEF. For this experiment, it was necessary to use cells that do not express endogenous p63RhoGEF, and we chose the HEK293 cells, which provide an additional benefit of easy transfection. Therefore, we over-expressed in HEK293 cells $p63RhoGEF^{331–580}$ with and without full-length p63RhoGEF. Due to competitive co-expression of full-length p63RhoGEF and $p63RhoGEF^{331–580}$, the expression level of full-length p63RhoGEF was higher when it was expressed alone. In spite

of this, immunoprecipitation resulted in precipitation of p63RhoGEF$^{331–580}$ only in the presence of full-length p63RhoGEF (Figure 6B). This finding is consistent with structural and *in vitro* studies suggesting that an intramolecular association of the PH domain with the DH domain results in autoinhibition [45]. We then examined whether the p63RhoGEF$^{331–580}$ interacts with $G\alpha_{q/11}$. Full-length p63RhoGEF and p63RhoGEF$^{331–580}$, both FLAG-tagged, were co-expressed with $G\alpha_{q/11}$wild-type or constitutively active $G_{\alpha 11}$ Q209T mutant in HEK293 cells (Figure 6C). As expected, $G\alpha_{q/11}$ Q209L showed higher affinity for full-length p63RhoGEF and p63RhoGEF$^{331–580}$ than $G\alpha_{q/11}$ wild-type, again, consistent with the *in vitro* studies showing direct association of activated $G\alpha_{q/11}$ and p63RhoGEF [45, 49].

Discussion

The RhoA/ROCK pathway serves as a final signaling hub in all SM tissues, accepting stimulatory signals initiated by diverse agonists, and activating Ca^{2+}-sensitization *via* inhibition of myosin light chain phosphatase. This physiological phenomenon is of critical significance in control of blood pressure, airway resistance, erectile dysfunction, gut motility, bladder and ureter function, etc [3]. The central role of the ROCK kinase prompted a number of investigations into its potential as a drug target [50]. However, the ubiquitous distribution of ROCK reduces the clinical potential of this approach, as it lacks the desired tissue specificity. On the other hand, downstream of ROCK, RhoA appears to be activated *via* diverse pathways, each utilizing a specific combination of GPCRs, trimeric G-proteins and GEFs. This raises the exciting possibility of selective targeting of tissue-specific GEFs for therapeutic use.

In order to better understand the intricate nature of the Ca^{2+}-independent component of contractile stimulation in SM, it is necessary to dissect the molecular pathways and identify the individual GPCRs as well as their partner G-proteins and coupled GEFs. Most vasoconstrictors, such as angiotensin II, ET-1, phenylephrine and thromboxane A2 bind to GPCRs coupled either to $G\alpha_{q/11}$ or $G\alpha_{12/13}$ or both[30]. In mice, knockout of $G\alpha_{q/11,}$ but not $G\alpha_{12/13}$, results in a fall in basal blood pressure while both are needed for the development of salt-induced hypertension. Studies of contractility of aorta from these mice showed that phenylephrine-induced force was completely abolished in the $G\alpha_{q/11}$ deficient mice but unaffected in the absence of $G\alpha_{12/13}$. On the other hand, contractions in response to ET-1 or U46619 are mediated by both G-proteins, with $G\alpha_{q/11}$ dominating for ET-1 and $G\alpha_{12/13}$ for U46619. The key question is what portion of Ca^{2+}-sensitized contractile response is transduced by each of these G-proteins, and if their signaling pathways are routed through distinct GEFs.

While $G\alpha_{12/13}$ was shown to activate RhoA through the three RGSL-containing, RhoA specific GEFs, i.e. PDZRhoGEF, LARG and p115RhoGEF [24], the pathways involving $G\alpha_{q/11}$ remained unclear. It has been established, that the RhoA-specific exchange factor p63RhoGEF is selectively activated by $G\alpha_{q/11}$ [45, 49, 51].. Recently, mRNA transcripts for p63RhoGEF were identified in rat aorta [52]. In an unrelated study, p63RhoGEF coupling through $G\alpha_{q/11}$ has been shown in cultured rat aortic SM cells to mediate angiotensin II signaling [35]. It was found that angiotensin II first induces a rapid influx of intracellular [Ca^{2+}] and activation of p63RhoGEF, followed by a significantly slower, [Ca^{2+}]/Janus kinase 2-dependent activation of p115RhoGEF to sustain RhoA signaling. However, the role of this pathway in contractility was assessed only by a collagen contraction assay using cultured rat aortic SM cells known to de-differentiate in culture. As expected, basal contraction increased upon angiotensin II pathway stimulation, but not in p63RhoGEF depleted cells [35].

The purpose of our investigation was to dissect the specific role of p63RhoGEF mediated Ca^{2+}-sensitization in blood vessels in response to stimulation *via* $G\alpha_{q/11}$, but through receptors other than that activated by angiotensin II. Our results confirm the presence of p63RhoGEF mRNA transcripts and protein in SM tissues and cells. Importantly, we show that expression profiles of upstream and downstream partners of p63RhoGEF (i.e. $G\alpha_{q/11}$ and RhoA) correlate well with p63RhoGEF across the tissues screened. We demonstrate for the first time that p63RhoGEF plays an important part in vessel contractility induced by ET-1, coupled primarily to $G\alpha_{q/11}$ through the ET_A receptors, but not by U46619, a thromboxane A2 analogue, known to couple predominately by $G\alpha_{12/13}$. The $G\alpha_{q/11}$ coupling leads to PLCβ activation, production of IP_3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol) and consequently increased intracellular $[Ca^{2+}]$ as well as activation of the RhoA pathway. To evaluate if the Ca^{2+}-sensitization pathway is affected by the knock-down of p63RhoGEF, we used α-toxin permeabilization, a method that retains agonist-GPCR coupling to RhoA under conditions where intracellular $[Ca^{2+}]$ is clamped. Ca^{2+} sensitization of contractile force induced by ET-1 but not by U46619 was significantly reduced when p63RhoGEF expression was suppressed (Figure 4). Furthermore, this was accompanied by a decrease in MYPT1 phosphorylation indicative of decreased RhoA/ROCK activity. These and other findings [30] lead us to suggest that the $G_{\alpha q/11}$ contribution to the regulation of basal and stimulated blood pressure may signal through p63RhoGEF rather than solely through PLCβ-induced Ca^{2+} release. Consistent with this idea is the observation that ROCK inhibitor Y-27632 reduces hypertension[53, 54] and that the Rho/ROCK signaling pathway in SM cells is an important contributor to peripheral resistance [55]. Significantly, p63RhoGEF mRNA is expressed in the resistance vessels, thoracodorsal and mesenteric arteries (Figure 1). P63RhoGEF protein was also detected in these vessels (data not shown). The contribution of this RhoGEF to the contractile behavior of resistance vessels will be explored in the future.

We also wondered if the *in vivo* mechanism by which p63RhoGEF operates in SM is consistent with a model derived from crystallographic studies of its complex with activated $G\alpha_{q/11}$ [49]. We hypothesized, that if the Ca^{2+}-sensitization effect is mediated by the same mechanism, then the isolated, recombinant extended PH domain ($p63RhoGEF^{331–580}$) should interfere with the process, either by binding to the activated $G\alpha_{q/11}$ and blocking downstream signaling, or by binding to the transiently open, i.e. active conformation of endogenous p63RhoGEF. To test this hypothesis in blood vessels, we performed contractility assays using β-escin permeabilized rabbit portal vein to allow introduction of the p63RhoGEF fragment into the SM cells. As expected, $p63RhoGEF^{331–580}$ induced relaxation following phenylephrine-induced contraction, which is known to be mediated by $G\alpha_{q/11}$[30]. This was accompanied by a decrease in RhoA-GTP and phospho-RLC_{20} suggesting suppression of $G\alpha_{q/11}$-coupled RhoGEF activity. Reduction of RhoA activity upon stimulation by phenylephrine in the presence of exogenous $p63RhoGEF^{331–580}$, acting as a dominant negative, was also observed in MEF cells. Overexpressed $p63RhoGEF^{331–580}$ associated with the full-length p63RhoGEF and with activated $G\alpha_{q/11}$. Our findings are consistent with the DH domain of p63RhoGEF being auto-inhibited through its extended PH domain under basal conditions with agonist activated $G\alpha_{q/11}$ relieving this auto-inhibition as shown in compelling *in vitro* experiments [45, 49]. A similar fragment of p63RhoGEF, comprising residues 295 to 580, was also shown to interfere with the angiotensin II mediated RhoA activation pathway in SM cells [35]. Taken together, these data strongly support the notion that the molecular mechanism inferred from the crystallographic studies is representative of the *in vivo* phenomenon.

In summary, the evidence presented in our paper shows that activation of p63RhoGEF by agonists that stimulate receptors coupled to $G\alpha_{q/11}$ leads to Ca^{2+} sensitization in blood vessels and cultured SM cells. We show that in addition to angiotensin II [35], both

phenylephrine and ET-1 also signal through p63RhoGEF to active RhoA and Ca^{2+} sensitized force. We conclude that different $G\alpha_{q/11}$ coupled receptors can signal through p63RhoGEF to contribute to vascular tone, and that it is not unique to angiotensin II stimulation. Indeed, angiotensin II plays a major role in hypertension while the tonic discharge of catecholamines by vasoconstricting sympathetic nerves maintains normotensive blood pressure. Further identification of specific RhoGEFs and characterization of their function in SM is a fertile ground for finding new targets that mediate specific functions of normal and diseased SM including the regulation of vascular tone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

DH	Dbl-homology
ET-1	Endothelin-1
GEF	Guanine nucleotide exchange factor
GPCR	G protein coupled receptor
HEK	Human embryonic kidney
MEF	Mouse embryonic fibroblast
MYPT1	Myosin targeting phosphatase subunit
PH	Pleckstrin-homology
RLC_{20}	Myosin regulatory light chain
ROCK	Rho-kinase
shRNA	short hairpin RA
SM	Smooth muscle

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Novelty and Significance

What is known?

- The small GTPase RhoA is activated by multiple agonists and significantly contributes to vascular contractility under physiological as well as pathophysiological conditions such as hypertension.
- Multiple GTP exchange factors (GEFs) are expressed in smooth muscle raising the possibility that specific agonists of specific G-protein coupled receptors (GPCRs) may be associated with distinct RhoGEFs.
- Angiotensin II has been shown to signal through $G\alpha_{q/11}$ in cultured smooth muscle cells but the role of other agonists in intact blood vessels is unknown, particularly agonists that regulate basal vascular tone.

What new information does this article contribute?

- p63RhoGEF is selectively activated by agonists such as α-adrenergic and endothelin-1 that signal through $G\alpha_{q/11}$ in blood vessels and maintain normotensive blood pressure.
- Knock down of p63RhoGEF decreases RhoA activity leading to increased myosin phosphatase activity, decreased myosin phosphorylation and decreased force development in blood vessels.
- We demonstrate *in vivo* that the molecular mechanism of action of p63RhoGEF is consistent with a model derived from crystallographic studies.
- p63RhoGEF is a potential selective therapeutic target for decreasing peripheral resistance and blood pressure.

Multiple agonists signaling through GPCRs activate the RhoA pathway which plays a central role in the regulation of basal tone, peripheral resistance, hypertension and cerebral vasospasm. RhoGEFs function upstream of RhoA, but it is unclear whether the multiple RhoGEFs present in smooth muscle (SM) couple selectively to specific agonists through GPCRs. If so, this would allow for selective therapeutic targeting of specific RhoGEFs in vascular SM. p63RhoGEF selectively coupled $G\alpha_{q/11}$, but not $G\alpha_{12/13}$, to RhoA activation in blood vessels, mediating the physiologically important Ca^{2+} sensitization of force induced with $G\alpha_{q/11}$ coupled agonists. Experiments using a recombinant extended PH domain fragment of p63RhoGEF show that this region serves as an autoinhibitory domain, interfering with the regulatory pathway by sequestering activated $G\alpha_{q/11}$. Our study extends the reported findings on angiotensin II to other $G\alpha_{q/11}$coupled agonists such as α-agonists and endothelin-1. Angiotensin II plays a major role in hypertension while catecholamines maintain normotensive blood pressure. We suggest that signaling through p63RhoGEF contributes to selective regulation of blood pressure. Further identification of specific RhoGEFs and characterization of their function in SM could identify new targets for regulating specific functions of normal and diseased SM.

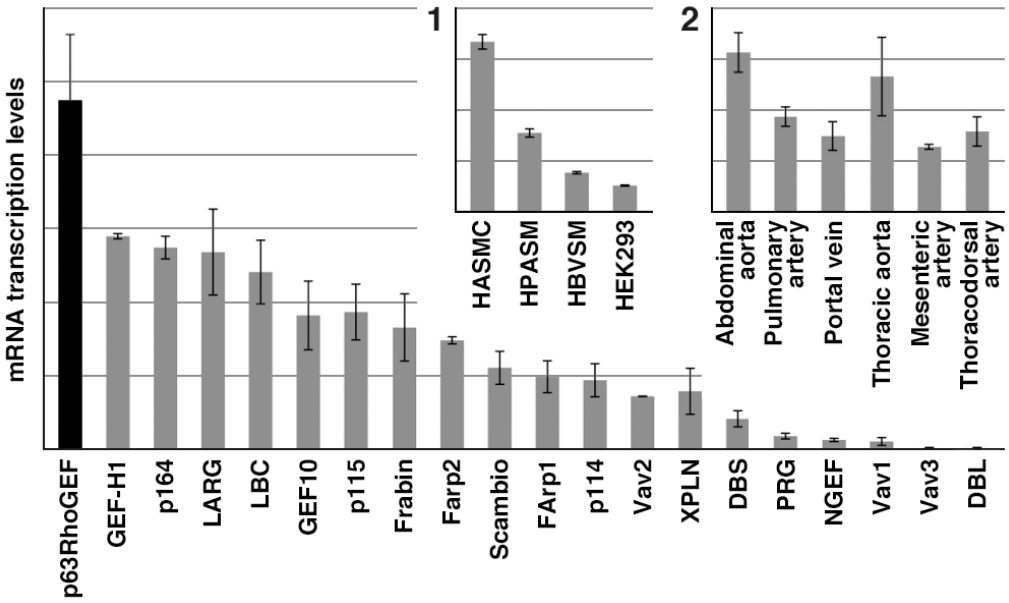

Figure 1. GEF mRNA transcription profile in mouse portal vein and p63RhoGEF mRNA expression levels in different human cell lines (inset 1) and mouse smooth muscle tissues (inset 2)

Human cell lines (inset 1): HASMC: aorta SM; HPASMC: pulmonary artery SM; HBVSMC: brain vascular SM; HEK293: human embryonic kidney cells.

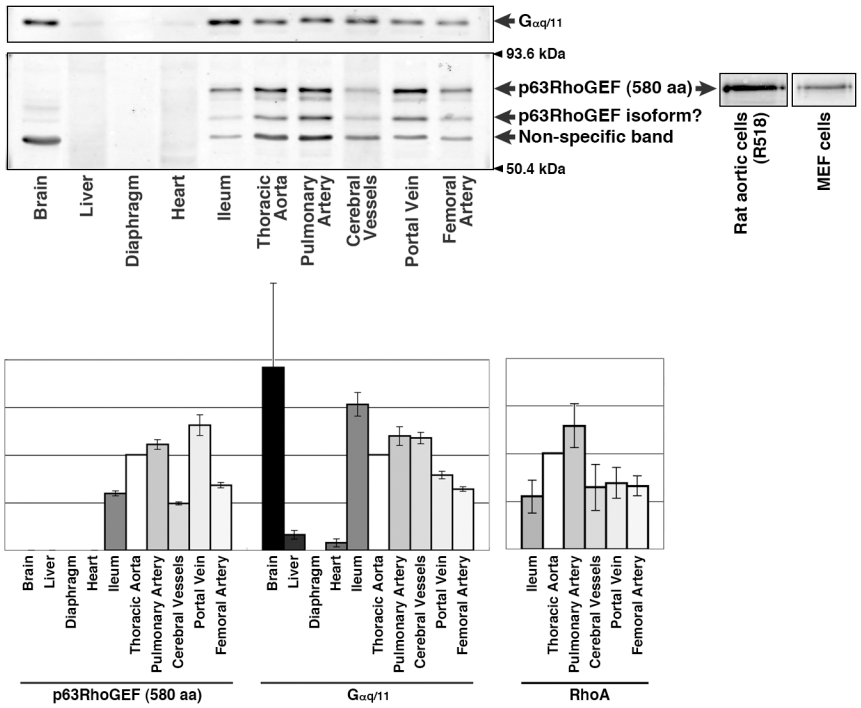

Figure 2. p63RhoGEF, its upstream effecter $G_{\alpha q/11}$ and downstream effecter RhoA in a variety of rat tissues and cultured cell lines

p63RhoGEF, $G_{\alpha q/11}$ and RhoA proteins are expressed in a variety of rat blood vessels and ileum. R518 rat aortic cells and MEF cells used in this study express p63RhoGEF. Protein expression is quantitated by Western blot analysis and levels normalized to total protein. Relative expression levels are presented arbitrarily.

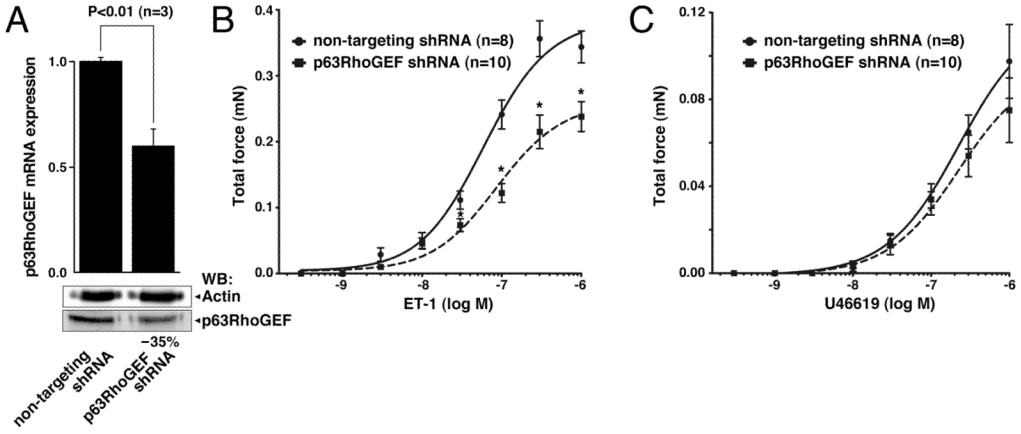

Figure 3. Suppression of p63RhoGEF expression significantly inhibits ET-1- but not U46619-induced force in intact portal veins

A, Knock-down of p63RhoGEF mRNA (n = 3) and protein (Western blotting of pooled sample from two mice) in mouse portal veins treated with a viral construct to express shRNA targeting p63RhoGEF as compared to the ones treated with a non-targeting control. **B, and C,** Force dose response curves to ET-1 (B) or U46619 (C) in intact mouse portal veins treated with viral constructs to express shRNA targeting p63RhoGEF or a non-targeting control shRNA. Shown are mean values ± S.E. * $P < 0.02$ versus non-targeting control. n = 8–10.

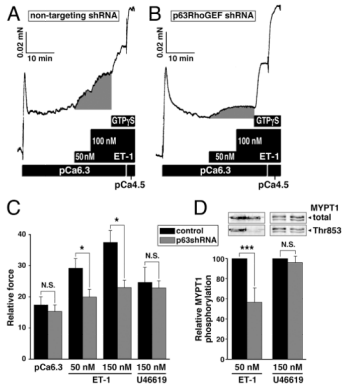

Figure 4. Suppression of p63RhoGEF expression significantly inhibits ET-1-induced Ca^{2+}-sensitized force and MYPT1 Thr853 phosphorylation, which is not observed in U46619 stimulation in permeabilized portal veins

A and B, ET-1- and GTPγS-induced force responses in permeabilized mouse portal veins following treatment with shRNA targeting p63RhoGEF (B) or a non-targeting control (A). [Ca^{2+}] has been clamped to pCa 6.3. Note that ET-1 Ca^{2+} sensitized force (shaded component) is inhibited by partial silencing of p63RhoGEF. GTPγS–induced force is a measure of the residual component of Ca^{2+} sensitization. pCa 4.5: maximal force. **C**, Summary of changes in ET-1 (50 nM and subsequent addition of 100 nM; i.e. 150 nM final concentration) induced Ca^{2+} sensitized force as shown in panels A and B and in U46619 (150 nM) induced Ca^{2+} sensitized force as in the same experimental scheme in panels A and B. The reduction in force, normalized to maximal force induced by pCa 4.5, for 50 nM ET-1 (IC_{50}) was 29.0±3.4 vs 19.7±2.6 respectively and at maximal ET-1 (150 nM) was 38.1±4.0% vs 23.0±2.5% respectively; no significant change in U46619-induced force between control and treated samples. Shown are mean values ± S.E. n = 12–14, * $P < 0.01$. **D**. Summary of changes in phosphorylation of the Rho kinase substrate MYPT1 at Thr853 following stimulation by ET-1 and U46619 in the Ca^{2+} sensitization protocol as detailed in Supplemental Material. Shown are mean values ± S.E. n = 4, *** $P < 0.02$.

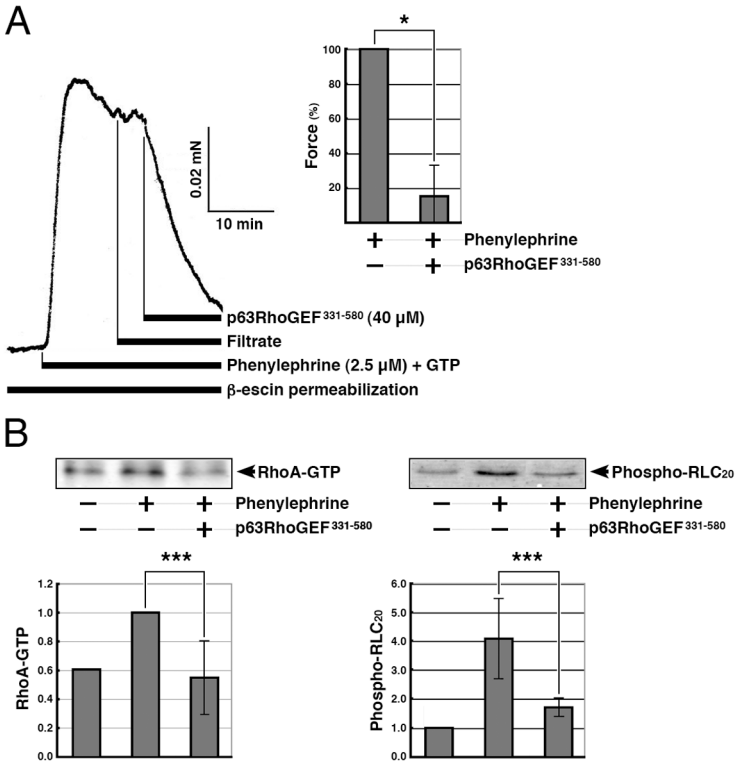

Figure 5. Suppression of phenylephrine-induced Ca^{2+} sensitized force, RhoA-GTP and phospho-RLC_{20} by p63RhoGEF$^{331–580}$ PH domain fragment in SM strips

A, Ca^{2+} sensitized force was induced by phenylephrine following β-escin-permeabilization to allow passage of p63RhoGEF recombinant protein across the cell membranes of rabbit portal vein SM strips. [Ca^{2+}] was buffered to pCa6.3. p63RhoGEF$^{331–580}$ PH domain fragment relaxed the phenylephrine-induced Ca^{2+} sensitized force. Shown are mean values ± S.E. n = 5, * $P < 0.01$. **B,** RhoA activity assay showing that addition of the p63RhoGEF$^{331–580}$ PH domain fragment reduced the level of RhoA-GTP and RLC_{20} phosphorylation induced by phenylephrine. The signals given by RhoA-GTP and Phospho-RLC_{20} are estimated by normalizing to total RhoA and total RLC_{20} respectively. Shown are mean values ± S.E. n = 3, *** $P < 0.04$. The sample number analyzed for the pre-stimulation control is 2 and therefore does not have a S.E.M.

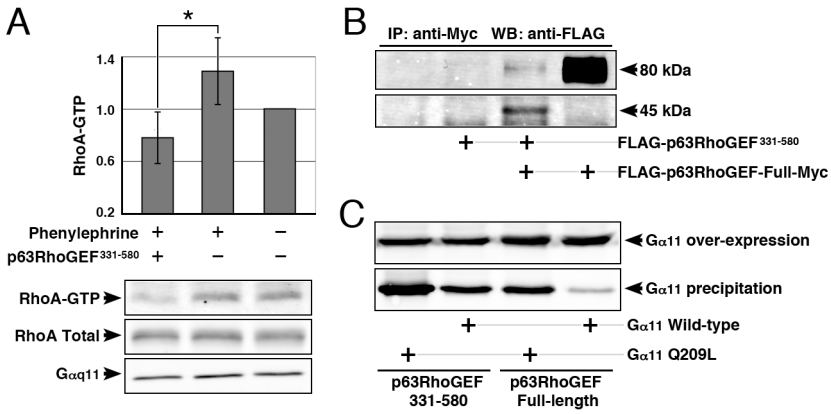

Figure 6. Suppression of RhoA-GTP by over-expression of p63RhoGEF$^{331–580}$ PH domain in MEF cells and interactions of p63RhoGEF$^{331–580}$ with full-length p63RhoGEF and wild-type and constitutively active $G_{\alpha 11}$

A, Western blot analysis and summary showing changes in RhoA-GTP before and after stimulation of MEF cells by phenylephrine (5 μM) in the presence and absence of exogenous p63RhoGEF$^{331–580}$ Shown are mean values ± S.E. n = 3, * $P < 0.01$. **B**, p63RhoGEF$^{331–580}$ alone or co-expressed with full-length p63RhoGEF in HEK293 cells, which lack endogenous p63RhoGEF. p63RhoGEF$^{331–580}$ (45 kDa) immunoprecipitated in the presence but not absence of full-length p63RhoGEF. **C**, Wild-type and constitutively active $G_{\alpha 11}$ (Q209L) expressed with FLAG-tagged p63RhoGEF$^{331–580}$ or FLAG-tagged full-length p63RhoGEF in HEK293 cells. Western blots of immunoprecipitation using anti-FLAG antibody show that $G_{\alpha 11}$ (Q209L) interacts more strongly with both p63RhoGEF$^{331–580}$ and full-length p63RhoGEF than wild type.