Proteinase-Activated Receptor 4 Activation Triggers Cell Membrane Blebbing through RhoA and β-Arrestin

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ABSTRACT
Proteinase-activated receptors (PARs) are a four-member family of G-protein-coupled receptors that are activated via proteolysis. PAR4 is a member of this family that is cleaved and activated by serine proteinases such as thrombin, trypsin, and cathepsin-G. PAR4 is expressed in a variety of tissues and cell types, including platelets, vascular smooth muscle cells, and neuronal cells. In studying PAR4 signaling and trafficking, we observed dynamic changes in the cell membrane, with spherical membrane protrusions that resemble plasma membrane blebbing. Since nonapoptotic membrane blebbing is now recognized as an important regulator of cell migration, cancer cell invasion, and vesicular content release, we sought to elucidate the signaling pathway downstream of PAR4 activation that leads to such events. Using a combination of pharmacological inhibition and CRISPR/Cas9–mediated gene editing approaches, we establish that PAR4-dependent membrane blebbing occurs independently of the Gaq/11- and Gα12,13-signaling pathways and is dependent on signaling via the β-arrestin-1/2 and Ras homolog family member A (RhoA) signaling pathways. Together these studies provide further mechanistic insight into PAR4 regulation of cellular function.

SIGNIFICANCE STATEMENT
We find that the thrombin receptor PAR4 triggers cell membrane blebbing in a RhoA– and β-arrestin–dependent manner. In addition to identifying novel cellular responses mediated by PAR4, these data provide further evidence for biased signaling in PAR4 since membrane blebbing was dependent on some, but not all, signaling pathways activated by PAR4.

Introduction
Proteinase-activated receptors (PARs) are a four-member family of G-protein–coupled receptors (GPCRs). PARs are unique among GPCRs because they are activated via proteolytic unmasking of a receptor-activating “tethered ligand” that interacts intramolecularily with the orthosteric ligand binding pocket to trigger signaling (Ramachandran et al., 2012). PAR4, the most recently identified member of this family (Xu et al., 1998), is expressed in a variety of tissues and cell types, including the platelets, vascular smooth muscle cells, neuronal cells, and some cancer cells.

Much work has been done to develop PAR1- and PAR4–targeted compounds as antiplatelet agents. Both PAR1 and PAR4 are expressed in human platelets, and both of these receptors are activated by the coagulation cascade enzyme thrombin. Importantly, although PAR1 and PAR4 appear to serve different roles in the platelet activation process (Kahn et al., 1999), with PAR1 as the high-affinity thrombin receptor playing an initiating role and the lower-affinity thrombin receptor PAR4 serving to consolidate and propagate the clot (Kahn et al., 1999). The PAR1 antagonist vorapaxar (ZONTIVITY), although highly effective in reducing cardiovascular complications, exhibited significant side effects with an elevated risk for bleeding, including in the brain (Morrow et al., 2012). This has spurred recent efforts to target PAR4. Recent work with small-molecule PAR4 antagonists has supported the idea that PAR4 antagonists are effective in reducing platelet-rich thrombus formation in human platelets ex vivo and in rodent and nonhuman primate models in vivo (Vong et al., 2017). In nonhuman primate models, PAR4 blockade was associated with low bleeding liability and had a markedly wider therapeutic

Abbreviations: BRET, bioluminescent resonance energy transfer; Cas9, CRISPR-associated protein 9; DMB-forskolin, 7-β-Deacetyl-7-[γ-(morpholino-butyryl)] forskolin; dRS-PAR4, mutant PAR4 receptor with an eight-amino acid C-terminal deletion; GPCR, G-protein–coupled receptor; HEK-293, human embryonic kidney 293 cells; KO, knockout; PAR, proteinase-activated receptor; PKC, protein kinase C; PTX, pertussis toxin; RhoA, Ras homolog family member A; RLuc, Renilla luciferase; ROCK, Rho-associated protein kinase; SHR, spontaneously hypertensive; SMC, vascular smooth muscle cells; WKY, Wistar Kyoto; YFP, yellow fluorescent protein.
in keeping with emerging literature for other GPCRs, we now know that activated PAR4 can directly couple to multiple G-protein–signaling pathways, including the Goαq/11 and Goq12/13 pathway (Woulfe, 2005; Kim et al., 2006), but is thought not to engage Goαq-dependent signaling pathways (Kim et al., 2006). PAR4 can also recruit and signal through β-arrestins (Li et al., 2011; Ramachandran et al., 2017). In recent work, we identified a C-terminal motif in PAR4 signaling through the Goαq/11 calcium signaling pathway and for recruiting β-arrestin-1/2 (Ramachandran et al., 2017). The mutant receptor with an eight–amino acid C-terminal deletion (dRS-PAR4) failed to internalize after activation with the PAR4 agonists thrombin or AYPGKF-NH2, suggesting a role for β-arrestins in PAR4 trafficking. A peptiducin targeting this C-terminal motif was also effective in attenuating PAR4-dependent platelet aggregation and thrombosis in vivo (Ramachandran et al., 2017). These recent findings point to the exciting possibility that it might be possible to therapeutically target PAR4 signaling in a pathway-specific manner. The present study was spurred by our observations that PAR4 activation rapidly triggered the formation of dynamic membrane blebs, which were absent in dRS-PAR4–expressing cells. Nonapoptotic plasma membrane blebbing is now recognized as a feature of various cellular processes, including directional cellular migration during development, cancer cell migration and invasion, neuronal cell remodeling, and vesicular content release (Charras, 2008; Charras and Paluch, 2008; Charras et al., 2008). Blebs are formed when the plasma membrane transiently detaches from the underlying actin filaments, resulting in intracellular pressure-mediated spherical membrane protrusions (Charras et al., 2008; Tinevez et al., 2009). The reassembly of actin filaments limits the expansion of blebs and actin polymerization while actomyosin contraction drives the retraction of the blebs (Charras et al., 2008). The molecular signals that trigger the formation of membrane blebs are beginning to be understood and include signaling from cell surface receptors such as GPCRs and receptor tyrosine kinases (Hagmann et al., 1999; Lawrenson et al., 2002; Godin and Ferguson, 2010; Chen et al., 2012; Laser-Azogui et al., 2014). A role for multiple Rho isoforms has also been described in regulating various aspects of bleb formation and retraction (Pinner and Sahai, 2008; Aoki et al., 2016; Gong et al., 2018). Previous work has described regulation of Rho signaling by GPCRs in both a G-protein– and β-arrestin–dependent manner (Sah et al., 2000; Barnes et al., 2005; Anthony et al., 2011).

Here, we examine in detail the pathways leading from PAR4 to the formation of membrane blebs. We find that inhibition of Goαq/11 signaling had no effect on the formation of PAR4–triggered membrane blebs, whereas blockade of β-arrestin-1/2– or Rho-dependent signaling significantly reduced blebbing.

Materials and Methods

Materials. Unless otherwise noted, all chemicals were purchased from Thermo Fisher (Waltham, MA). Peptide ligands were custom synthesized by Genscript (Piscataway, NJ) at greater than 95% purity. Thrombin was purchased from Calbiochem (Oakville, ON), and coelenterazine-h was from Nanolight Technology (Pineport, AZ). All antibodies (anti-β-arrestin-1/2, anti-RhoA, anti-actin, anti-rabbit–Horseradish Peroxidase) used in this study were purchased from Cell Signaling Technologies. YM254890 was from Wako chemicals (Richmond, VA), GSK269962 was from Tocris (Oakville, ON), and all other chemicals were from Sigma-Alrdrich (Oakville, ON).

Cell Culture and Transfections. Human Embryonic Kidney 293 (HEK-293; ATCC) cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco) with 10% FBS (Gibco), 1% penicillin-streptomycin (Gibco), and 1% sodium pyruvate (Gibco). Cells stably expressing PAR4-YFP or dRS-PAR4-YFP were maintained in the above media supplemented with 600 μg/ml of genetin (Gibco). dRS-PAR4–YFP–expressing HEK-293 cells have been previously described (Ramachandran et al., 2017). PAR1-KO-PAR4-YFP-HEK-293 (Mihara et al., 2016) and β-arrestin-1/2-KO HEK-293 cells (Thibeault et al., 2020) have been previously characterized. Cells were transiently transfected using a modified calcium phosphate method (Ferguson and Caron, 2004). Experiments were performed 48 hours posttransfection. Primary rat smooth muscle cells were isolated and cultured as previously described (Gros et al., 2006).

Constructs. PAR4-YFP and dRS-PAR4-YFP have been previously described (Ramachandran et al., 2017). Dominant negative Goαq, Go12Q231L/D299N (plasmid # GNA12000X0) and Goαq, Go13Q226L/D294N (plasmid # GNA13000X0) were obtained from the cDNA resource center (www.cdna.org) (Yang et al., 2005; Lauckner et al., 2008; Goupil et al., 2010). pGloSensor-22F eAMP (Promega) and pcDNA3.1–α–2 adrenergic receptor plasmids were a kind gift from Dr. Peter Chidiac. GFP-NS-PKC/γ (PKC-GFP) was a gift from Tobias Meyer (plasmid # 21204; Addgene).

Creation of RhoA Knockout Cells. Rhoa HEK-293 cells (Rhoa-KO HEK) were generated using CRISPR/Cas9–associated protein 9 (Cas9)–mediated gene targeting. Guides targeting Rhoa were designed using a web-based design tool as previously described (Cong et al., 2013; Hsu et al., 2013). Gene-specific guides for Rhoa (CGAGTTTGGCATCG CGGAC, CGTGTCGGGAGTCGCAAAC, GAGTTCGACGCTTTGCG CC, GACTCGGGACCCGCTTCC) were ligated into the PX458 vector (a kind gift from Dr. Feng Zhang, MIT, plasmid # 48183; Addgene), verified by direct sequencing, and transfected in HEK cells via the calcium phosphate method. At 48 hours posttransfection, GFP–expressing single cells were flow-separated into individual wells of a 96-well plate (Becton Dickinson FACSARIA III). Clonal cells from individual wells were screened by Western blotting to identify cell lines that were deficient in Rhoa (Supplemental Fig. 1).

Confocal Microscopy. Cells were plated onto glass-bottom 35-mm dishes (MatTek, Ashland, MA) and imaged using a Zeiss confocal Laser Scanning Microscope 510 Meta Non-Linear Optics. Yellow fluorescent protein was excited with 514 laser line and visualized with 535–560 filter set. mCherry fluorophore was excited with 543 laser line and visualized with 560–590. GFP was excited with 488 laser line and visualized with 530–560 filter set. Cell shape change experiments were conducted as follows. HEK-293 cells stably expressing C-terminally enhanced yellow fluorescent protein–tagged PAR4, CRISPR/Cas9 knockout cell lines, or transiently transfected cells were plated into three to four glass-bottom dishes and subsequently treated with vehicle or inhibitor, as indicated in the figure legends. Plates were then placed on a heated stage on the microscope, and PAR4–YFP was activated with AYPGKF-NH2 (30 μM) or thrombin (3 Units/ml), as indicated in the figure legends. Over 10 minutes, 6–12 images per dish were taken. This represented one independent experiment and was repeated three to five times on different days with newly cultured cells. For experiments that used stably expressing cell lines or stably knockout cell lines, an experiment was considered an independent replicate when it was conducted on a different day with cells that were cultured on different days. Images were scored twice, once by the experimenter and once by a blinded individual, and statistical analysis was used to ensure that scoring was not different between observers. In all cases, there were no significant differences between.
the observer scores. Cells were scored by manually counting the number of cells displaying membrane protrusions (indicated by arrows in the figures) versus cells that did not display any membrane protrusions. The “degree” of blebbing was not studied here.

**Bioluminescent Resonance Energy Transfer Assay for β-Arrestin-1/2 Recruitment.** Bioluminescent resonance energy transfer was measured between C-terminally YFP-tagged PAR4 (Ramachandran et al., 2017) and Renilla luciferase (RLuc)-tagged β-arrestin-1 or β-arrestin-2 (a kind gift from Dr. Michel Bouvier, U. de Montreal) after 20 minutes of receptor activation, as described in previous studies (Ramachandran et al., 2009). Briefly, PAR4-YFP (1 μg) and β-arrestin-1-Rlu or -2-Rlu (0.1 μg) were transiently transfected in cells plated in a six-well plate for 24 hours. Cells were replated into white 96-well culture plates and cultured for a further 24 hours. Interactions between PAR4 and β-arrestin-1/2 were detected by measuring the bioluminescent resonance energy transfer (BRET) ratio at timed intervals over 20 minutes after the addition of h-coelenterazine (5 μM; Nanolight Technology) on a Mithras LB940 plate reader (Berthold Technologies) with the appropriate BRET filters.

**gloSensor cAMP Assay.** HEK-293 cells were transfected with α2A adrenergic receptor (1 μg) and pGloSensor-22F cAMP sensor (1 μg) plasmids using the X-tremeGENE 9 transfection reagent (Sigma). Cells were plated into a white 96-well plate (Corning) 24 hours after transfection along with either vehicle control or pertussis toxin (PTX, 100 ng/ml) and incubated overnight (approximately 18 hours prior to experiment). Forty-eight hours posttransfection, cell media were replaced with minimal essential medium (no phenol red) supplemented with bovine serum albumin (0.1% w/v), D-luciferin (2 mM), and HEPES (20 mM) for 2 hours. At 15 minutes prior to measurement, 3-isobutyl-1-methylxanthine (200 μM) was added into wells. α2A agonist 5-Bromo-6-(2-imidazolin-2-ylamino)quinoxaline tartrate (UK 14, 304, 1 nM) was added 5 minutes prior to measurement. 7-β-Deacetyl-7 β[γ-(morpholino-butyryl)] butyryl forskolin (DMB-forskolin, 2 μM) was added to each well to stimulate cAMP production. Luminescence was recorded every 2.5 minutes for 30 minutes on a Mithras LB940 plate reader (Bethold Technologies), and increase in luminescence over time was graphed. These experiments were conducted to determine the efficacy of the pertussis toxin lot and concentration used in both BRET and confocal experiments (Supplemental Fig. 2).

**Statistical Analysis.** All data shown are presented as means with S.D. Statistical analysis of data and curve fitting were done with Prism 7 software (GraphPad Software, San Diego, CA). Statistical tests are listed in the figure legends. For studies done with inhibitors, a paired t test was used since the cell population is the same for both vehicle- and drug-treated conditions. For studies comparing different cell lines, an unpaired t test was used, since observations were from different cell populations. Finally, data were tested for normality using a Shapiro-Wilk test, and for data that were not normally distributed (nonparametric), a subsequent nonparametric test was used to determine significance.

**Results**

**Activation of PAR4 Elicits Cell Shape Changes that Are Dependent on a C-Tail Eight-Amino Acid Sequence.** Stimulation of plasma membrane–localized PAR4-YFP in HEK-293 cells stably expressing PAR4-YFP (Fig. 1A) with the PAR4-specific peptide agonist (AYPGKF-NH2) resulted in cell shape changes that resembled membrane protrusions and are illustrated in Fig. 1. PAR4-expressing cells treated with AYPGKF-NH2 (30 μM) displayed protrusions forming at the plasma membrane, indicated by arrows (Fig. 1B). These structures resemble membrane blebs and began to form around 2 minutes post–agonist stimulation.

![Fig. 1](image-url) - Activation of PAR4 mediates a cell shape change response. Representative confocal micrographs showing HEK-293 cell stably expressing PAR4-YFP (A and B). Cells were treated with AYPGKF-NH2 (30 μM) for 2 minutes prior to imaging. Size bars are 20 μm, and arrows indicate membrane-membrane protrusions (blebs). HEK-293 cells stably expressing PAR4-YFP (C–E) were incubated in either DMSO (C) or blebbistatin (10 μM) (B) for 15 minutes prior to a 2-minute treatment with AYPGKF-NH2 (30 μM) followed by confocal microscopy imaging. Arrows show bleb formation; size bars are 20 μm. (E) Percentage of cells stably expressing PAR4-YFP that displayed membrane blebbing in response to AYPGKF-NH2 (30 μM) treatment in the presence of either DMSO or blebbistatin. Numbers above the bars indicate the total number of cells scored for blebbing response. Data represent mean ± S.D. of four independent experiments, and the asterisk shows significant differences; paired t test, P < 0.05.
and lasted for up to 30 minutes in the presence of AYPGKF-NH$_2$ (30 μM). To further verify that the cytoskeletal changes were indeed membrane blebs, we examined the effect of treating cells with blebbistatin, a small-molecule inhibitor of myosin II ATPase (Cheung et al., 2002; Straight et al., 2003), which functions by locking actin heads in a low–actin affinity complex (Kovács et al., 2004) and is reported to inhibit non-apoptotic membrane blebbing. Incubation of cells with blebbistatin significantly reduced the AYPGKF-NH$_2$-stimulated membrane bleb response in PAR4-YFP-expressing HEK-293 cells (15.37% ± 8.29%) (Fig. 1, D and E). In contrast, PAR4-YFP-expressing cells treated with vehicle control (DMSO) (Fig. 1C) displayed membrane blebs with a mean of 82.25% ± 16.47%. In a recent study, we described a mutant PAR4 receptor lacking eight amino acids from the C-tail, dRS-PAR4-YFP (Ramachandran et al., 2017). We observed that in contrast to the wild-type receptor, dRS-PAR4-YFP-expressing cells displayed significantly less blebbing in response to AYPGKF-NH$_2$ (30 μM) treatment (Fig. 2, A–C). HEK-293 cells stably expressing wild-type PAR4-YFP displayed membrane blebbing (82.33% ± 2.08%), as opposed to 8.2% ± 4.07% dRS-PAR4-YFP-expressing cells, indicating that PAR4-triggered membrane blebbing required the activation of signaling pathways that are dependent on the eight–amino acid sequence in the C-tail of PAR4. Previously, we established that dRS-PAR4-YFP does not couple to G$_{q/11}$ and is unable to recruit β-arrestins in response to thrombin or AYPGKF-NH$_2$ activation (Ramachandran et al., 2017). Since this mutant receptor is also unable to activate blebbing, we hypothesized that PAR4 cell shape changes are G$_{q/11}$- and/or β-arrestin–dependent and examined the effect of blocking these pathways on bleb formation.

**PAR4-Mediated Cell Shape Change Is G$_{q/11}$- and G$_{q/11}$-Independent.** To determine whether PAR4-triggered blebbing is G$_{q/11}$-dependent, we treated cells with the potent and selective G$_{q/11}$ inhibitor YM254890 (Taniguchi et al., 2004). It is well established that GPCRs couple to G$_{q/11}$ to mobilize calcium and activate protein kinase C (PKC) (Exton, 1996; Wettachreuck and Offermanns, 2005). Activated PKC translocation from the cytosol to the plasma membrane can be observed to monitor this process (Dale et al., 2001; Policha et al., 2006). We employed this assay to confirm the efficacy of YM-254890 in inhibiting G$_{q/11}$ signaling through PAR4.

Cells were transiently transfected with PAR4-mCherry and PKC-GFP. PAR4 expression was observed at the cell membrane, and PKC expression was evident in the cytoplasm in resting cells (Fig. 3A). Upon treatment with AYPGKF-NH$_2$ (30 μM), PKC-GFP translocated to the membrane (Fig. 3B), and blebbing responses were observed, as before (Fig. 3C). In cells treated with YM254890 (100 nM), PKC-GFP failed to translocate to the membrane after treatment with AYPGKF-NH$_2$ (Fig. 3, D–F). YM254890 (100 nM)–treated cells, however, maintained their ability to bleb in response to AYPGKF-NH$_2$ (Fig. 3, D–F). The role of G$_{q/11}$ in PAR4-mediated blebbing was further quantified in HEK-293 cells stably expressing PAR4-YFP treated with either DMSO vehicle or YM254890 prior to stimulation with AYPGKF-NH$_2$ (30 μM). Blebbing in vehicle-treated cells was not significantly different from cells treated with YM254890: 81.0% ± 4.5% and 77.0% ± 2.5%, respectively (Fig. 3G). These data indicate that YM254890 functionally blocks G$_{q/11}$ signaling, as indicated by a lack of PKC translocation, but does not block cell shape changes mediated by PAR4 activation.

HEK-293 cells transiently expressing PAR4-mCherry with PKC-GFP showed that activation of PAR4-mCherry with AYPGKF-NH$_2$ causes a translocation of PKC-GFP from the cytosol (Fig. 4A) to the plasma membrane (Fig. 4B). In contrast, HEK-293 cells transiently expressing dRS-PAR4-mCherry with PKC-GFP showed that activation of dRS-PAR4-mCherry does not cause a redistribution of PKC-GFP to the membrane (Fig. 4, C and D). Although PKC activation is downstream of G$_{q/11}$, since dRS-PAR4 does not activate PKC and does not elicit cell shape changes, we tested PKC for a potential role in mediating PAR4-mediated cell shape changes. HEK-293 cells stably expressing PAR4-YFP were treated with the PKC inhibitor Gö6983 (Gschwendt et al., 1996) prior to stimulation with AYPGKF-NH$_2$ (30 μM) and visualization by confocal microscopy (Fig. 4, E and F). There was no significant difference in the number of vehicle-treated (DMSO) cells displaying blebbing when compared with cells treated with Gö6983 in response to AYPGKF-NH$_2$ (74.5% ± 4.6% and 72.2% ± 9.2%) (Fig. 4G). These data suggest, then, that neither G$_{q/11}$ nor PKC facilitates PAR4-mediated cell shape changes.

After ruling out G$_{q/11}$ as a potential signaling partner for PAR4-mediated membrane blebs, we tested G$_{q_1}$ recruitment as a potential regulator of these responses through inhibition of G$_{q_1}$ signaling with PTX. HEK-293 cells stably expressing...
PAR4-YFP were incubated with pertussis toxin (100 ng/ml) for 18 hours prior to stimulating the cells with AYPGKF-NH₂ (30 μM; Fig. 5, A and B). We did not observe any reduction in the number of cells that displayed blebbing when compared with cells incubated with vehicle control (saline) (76.3% ± 6.00% and 78.0% ± 8.14%, respectively) (Fig. 5C). Since we did not see an effect of PTX on membrane blebbing, we tested the efficacy of the toxin. To accomplish this, we employed

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**Fig. 3.** PAR4-mediated cell shape change is Go_{q/11}-independent. Confocal micrographs of HEK-293 cells transiently transfected with PAR4-mCherry (not shown) and with 1 μg of PKC-GFP (shown in black) were treated with DMSO followed by stimulation with AYPGKF-NH₂ (30 μM) (A–C) or treated with YM254890 (100 nM), a Go_{q/11} inhibitor, for 20 minutes prior to addition of AYPGKF-NH₂ (30 μM) (D–F). Arrows show bleb formation; size bars are 20 μm. (G) Percentage of cells transiently transfected with 1 μg each of PAR4-mCherry and PKC-GFP displaying membrane blebbing in response to AYPGKF-NH₂ (30 μM) in the presence of either DMSO (vehicle) or YM254890 (100 nM). Numbers above the bars indicate total number of cells scored. Data represent mean ± S.D. of three independent experiments; not significantly different, paired t test.

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**Fig. 4.** PAR4-mediated cell shape change is PKC-independent. Confocal micrographs of HEK-293 cells transiently transfected with 1 μg each of PAR4-mCherry (not shown) and PKC-GFP (shown in black) (A and B) or 1 μg each of dRS-PAR4-mCherry (not shown) with PKC-GFP (shown in black) (C and D) were treated with AYPGKF-NH₂ (30 μM) and imaged by confocal microscopy; size bars are 20 μm. HEK-293 cells stably expressing PAR4-YFP were incubated with DMSO (E) or with Go6983 (100 nM), a PKC inhibitor, for 15 minutes (F) prior to a 2-minute stimulation with AYPGKF-NH₂ (30 μM) and subsequent imaging by confocal microscopy. (G) Percentage of cells stably expressing PAR4-YFP displaying membrane blebbing in response to AYPGKF-NH₂ (30 μM) treatment in the presence of either DMSO (vehicle) or Go6983 (100 nM). Numbers above the bars indicate total number of cells scored. Data represent mean ± S.D. of four independent experiments; not significantly different, paired t test.
a luminescence-based assay using the pGloSensor-22F to measure the inhibition of cAMP production in response to agonist stimulation of the Ga_i-coupled α-2 adrenergic (α2A) receptor. Loss of α2A-stimulated, Ga_i-coupled inhibition of cAMP production by pertussis toxin–mediated inhibition of Ga_i (thus, no inhibition of cAMP production) indicates efficacy of pertussis toxin. In HEK-293 cells treated with DMB-forskolin to induce cAMP production, treatment of cells with pertussis toxin or with vehicle control showed no significant reduction on cAMP production, indicating that PTX does not reduce cAMP independently of Ga_i (Supplemental Fig. 2, A and C). Agonist stimulation of the Ga_i-coupled α2A receptor with UK 14, 304,

Fig. 5. PAR4-mediated cell shape change is Ga_i-independent. Confocal micrographs showing HEK-293 cells stably expressing PAR4-YFP were incubated with either DMSO (A) or (B) PTX (100 ng/ml), a Ga_i inhibitor, for 18 hours prior to AYPGKF-NH2 (30 μM) treatment of 2 minutes and subsequent confocal imaging. Arrows indicate bleb formation; size bars are 20 μm. (C) Percentage of cells stably expressing PAR4-YFP and displaying membrane blebbing in response to AYPGKF-NH2 (30 μM) in the presence of either DMSO (vehicle) or PTX (100 ng/ml). Numbers above the bars indicate total number of cells scored. Data represent mean ± S.D. of three independent experiments; not significantly different, paired t test.

Fig. 6. PAR4-mediated cell shape change is Ga_{12/13}, RhoA-, and ROCK-dependent. Confocal micrographs of HEK-293 cells stably expressing PAR4-YFP (shown in black) either alone (A) or transiently transfected with 0.5 μg each of Ga_{12/Q231L/D299N} and Ga_{13/Q226L/D294N} (B) or with 1 μg of Ga_{12/Q231L/D299N} (C) or 1 μg of Ga_{13/Q226L/D294N} (D) were treated with AYPGKF-NH2 (30 μM) for 2 minutes and subsequently imaged by confocal microscopy. Arrows indicate bleb formation, and size bars are 20 μm. (E) Percentage of cells stably expressing PAR4-YFP alone or with transiently expressing Ga_{12/Q231L/D299N} and/or Ga_{13/Q226L/D294N}, displaying membrane blebbing in response to AYPGKF-NH2 (30 μM). Numbers above the bars indicate total number of cells scored. Data indicate mean ± S.D. of three independent experiments, and asterisks indicate statistical difference; one-way ANOVA, P > 0.05. Confocal micrographs of HEK-293 cells (F) and RhoA-KO HEK (G) transiently transfected with 1 μg of PAR4-YFP were stimulated with AYPGKF-NH2 (30 μM) for 2 minutes prior to imaging. Arrows indicate bleb formation; size bars are 20 μm. (H) Percentage of HEK-293 or RhoA-KO HEK transiently expressing PAR4-YFP and displaying membrane blebbing in response to AYPGKF-NH2 treatment (30 μM). Data represent mean ± S.D. of four independent experiments, and asterisks show significant differences; unpaired t test, P < 0.05. Confocal micrographs of HEK-293 cells stably expressing PAR4-YFP and incubated with either DMSO (I) or GSK269962 (100 nM) for 1 hour (J) prior to a 2-minute treatment with AYPGKF-NH2 (30 μM) and subsequent imaging. Arrows show bleb formation, and size bars are 20 μm. (K) Percentage of PAR4-YFP stably expressing HEK-293 cells displaying membrane bleb formation in the presence of AYPGKF-NH2 (30 μM) with DMSO (vehicle) or GSK269962 treatment. Data represent mean ± S.D. of four independent experiments, and asterisks show significant differences; paired t test, P < 0.05. WT, wild-type.
induced an inhibition of DMB-forskolin–mediated cAMP production. G_{12}-coupled inhibition of cAMP production downstream of α_{2A} receptor activation was abolished by PTX treatment (Supplemental Fig. 2, B and C). Taken together, these data indicate that PTX was indeed able to inhibit G_{12} signaling as expected and that the lack in inhibition of PAR4-mediated blebbing in PTX-treated cells indicates that G_{12}-mediated signaling is not involved in this response.

PAR4-Mediated Cell Shape Change Is RhoA- and ROCK-Dependent. Having ruled out G_{q/11} and G_{q} as mediators of PAR4-activated membrane blebbing, we sought to explore the involvement of other G-proteins. G_{12/13} proteins are known to activate the small G-protein RhoA. RhoA has been implicated in actin cytoskeletal rearrangements mediated by other GPCRs, and thus, we tested its role in this pathway. To explore a role for G_{12/13} in PAR4 signaling, we employed the use of two dominant negative constructs: G_{12}Q^{231L/D^{299N}} and G_{13}Q^{238L/D^{294N}}. HEK-293 cells expressing PAR4 (Fig. 6A) were transiently transfected with G_{12}Q^{231L/D^{299N}} alone (Fig. 6C), G_{12}Q^{231L/D^{299N}} alone (Fig. 6D), or with both dominant negative constructs (Fig. 6B) and were imaged by confocal microscopy subsequent to AYPGKF-NH_{2} treatment. As before, we observed blebbing in PAR4-expressing cells (85.10% ± 7.08%), which was significantly reduced in cells expressing either G_{12}Q^{231L/D^{299N}} (52.45% ± 2.83%) or G_{13}Q^{238L/D^{294N}} and G_{13}Q^{238L/D^{294N}} (59.31% ± 8.66%) (Fig. 6E). However, expression of just G_{12}Q^{238L/D^{294N}} did not significantly the number of cells blebbing in response to AYPGKF-NH_{2} (61.93% ± 15.19%). These data support a model whereby PAR4 couples to G_{12} to elicit blebbing responses.

Since RhoA can be activated by G_{12} (Siehler, 2009), and since RhoA is also well established as a regulator of actin cytoskeleton rearrangements (Barnes et al., 2005; Aoki et al., 2016), we examined whether PAR4-mediated blebbing was dependent on RhoA and Rho-associated kinase (ROCK). We used CRISPR/Cas9 targeting to express this signaling pathway by creating a RhoA knockout HEK-293 cell line (RhoA-KO HEK). RhoA-KO HEK cells and control HEK-293 cells were transiently transfected with PAR4-YFP, stimulated with AYPGKF-NH_{2} (30 μM), and imaged by confocal microscopy (Fig. 6, F and G). In total, 75% ± 13.5% of control HEK-293 cells expressing PAR4-YFP and treated with AYPGKF-NH_{2} displayed membrane blebbing, whereas RhoA-KO HEK expressing PAR4-YFP show significantly fewer cells with blebs in response to agonist (22.8% ± 13.88%) (Fig. 6H). To further interrogate this pathway, we employed the ROCK inhibitor GSK269962. Treatment of PAR4-YFP–expressing HEK-293 cells with the ROCK-specific inhibitor GSK269962 (Stavenger et al., 2007) significantly reduced the number of blebbing cells (Fig. 6, I and J). In total, 72.53% ± 12.48% of DMSO vehicle control–treated cells displayed blebbing, whereas only 15.6% ± 5.37% of cells treated with GSK269962 (100 nM) displayed bleb formation in response to AYPGKF-NH_{2} (30 μM; Fig. 6K). Taken together, these data indicate that stimulation of PAR4-YFP elicits cell shape changes that are blocked by blebbistatin and by the ROCK inhibitor GSK269962. Further, these cell shape changes are significantly reduced in the presence of dominant negative G_{12} protein and do not occur in cells that do not express RhoA protein. Overall, these data suggest that PAR4 causes membrane blebbing through a G_{12}, RhoA, and ROCK-dependent pathway.

To confirm that these cellular responses can also be recapitated by a physiologic PAR4 agonist, we tested the ability of thrombin to elicit similar cell shape changes. Since thrombin can also activate PAR1, which is endogenously expressed in HEK-293 cells, we conducted these experiments in PAR1 knockout HEK-293 cells (PAR1-KO HEK) (Mihara et al., 2016) stably expressing PAR4-YFP (PAR1-KO-HEK-PAR4-YFP). PAR1-KO-HEK-PAR4-YFP cells were treated with 3 U/ml thrombin and visualized by confocal microscopy. Treatment of cells with thrombin caused cell shape changes similar to what was observed in HEK-293-PAR4-YFP cells treated with AYPGKF-NH_{2} (Fig. 7A). PAR1-KO-HEK-PAR4-YFP cells were then treated with blebbistatin prior to thrombin stimulation (Fig. 7B). Blebbistatin significantly reduced the number of PAR1-KO-HEK-PAR4-YFP cells displaying membrane blebs from 68.3% ± 8.08% to 11.67% ± 10.02% (Fig. 7D). Finally, PAR1-KO-HEK-PAR4-YFP cells were treated with GSK269962 prior to stimulation with thrombin (Fig. 7C). GSK269962 (100 nM) significantly reduced the number of cells that displayed blebbing in response to thrombin stimulation (9.33% ± 7.50%) (Fig. 7D). Together, these data indicate that cell blebbing is triggered not only by the synthetic PAR4-activating peptide AYPGKF-NH_{2} but also by one of the endogenous activators of PAR4, thrombin, in a PAR1-null background. Cell shape changes mediated by thrombin activation of PAR4 are also RhoA- and ROCK-dependent.

![Fig. 7. Thrombin-induced PAR4-mediated cell shape change is ROCK-dependent. Confocal micrographs showing control HEK-293 cells with CRISPR/Cas9 deletion of PAR1 (PAR1-KO-HEK-293) and stably expressing PAR4 (PAR1-KO-HEK-PAR4-YFP-HEK-293) incubated in either DMSO (A) or blebbistatin (10 μM) for 15 minutes (B) or GSK269962 (100 nM) for 1 hour (C) prior to a 2-minute treatment with thrombin (3 U/ml) and subsequent imaging. Arrows show bleb formation; size bars are 20 μm. (D) Percentage of PAR1-KO-HEK-PAR4-YFP-HEK-293 cells showing membrane blebbing in response to thrombin treatment (3 U/ml) in the presence of either DMSO (vehicle), blebbistatin (10 μM), or GSK269962 (100 nM). Numbers above the bars indicate the total number of cells scored. Data represent mean ± S.D. of four independent experiments, and asterisks show significant differences; one-way ANOVA, P < 0.05.](image-url)
PAR4-Mediated Cell Shape Change Is β-Arrestin-Dependent. Since the nonblebbing dRS-PAR4-YFP–expressing cells are also deficient in β-arrestin recruitment (Ramachandran et al., 2017), we next examined the contribution of β-arrestin-mediated signaling in PAR4-dependent cell membrane blebbing. To elucidate a role for β-arrestins in PAR4-mediated cell shape change, a β-arrestin-1 and 2 double knockout cell line (β-arrestin-1/2 KO HEK) was created using CRISPR/Cas9 targeting, β-Arrestin-1/2 KO HEK cells transiently expressing PAR4-YFP (β-arrestin-1/2 KO HEK-PAR4-YFP) were treated with AYPGKF-NH$_2$ (30 μM) and visualized by confocal microscopy (Fig. 8, A and B). A modest but significant reduction in the number of blebbing β-arrestin-1/2 KO HEK-PAR4-YFP (46% ± 18.34%) was observed when compared with control HEK-293 cells transiently expressing PAR4-YFP (80.8% ± 6.10%) (Fig. 8C). These results indicate that PAR4-elicited membrane blebbing is, in part, β-arrestin-dependent.

Since recent reports have questioned whether β-arrestin-mediated signaling can occur in the absence of G-protein recruitment to PAR4, we blocked G$_{q/11}$ using the inhibitor YM254890 (100 nM) and examined its ability to disrupt β-arrestin recruitment to PAR4. To this end, we blocked G$_{q/11}$ with the inhibitor YM254890 (100 nM) and blocked G$_{o}$ with pertussis toxin (100 nM) and visualized by confocal microscopy (Fig. 8, A and B). A modest but significant reduction in the number of blebbing by confocal microscopy (Fig. 8, A and B). A modest but significant reduction in the number of blebbing by confocal microscopy (Fig. 8D) treatment condition. Treatment of cells with pertussis toxin had no effect on β-arrestin recruitment at any of the concentrations tested (Fig. 8E).

Finally, we tested a role for G$_{q}$ signaling in PAR4-mediated cell shape change using the G$_{q}$ inhibitor gallein. HEK-293 cells stably expressing PAR4-YFP were incubated with gallein (10 μM) or vehicle control (DMSO) prior to treatment with AYPGKF-NH$_2$ (30 μM) (Fig. 9, A and B). Cells treated with gallein did not show a significant reduction in membrane blebbing compared with DMSO-treated cells (61.75% ± 9.75% and 85.28% ± 10.56%, respectively) (Fig. 9C). Cells expressing PAR4-YFP and either β-arrestin-1-Rluc or -2-Rluc incubated with gallein prior to treatment with AYPGKF-NH$_2$ at 30, 100, and 300 μM also retained their ability to recruit β-arrestin-1 and 2 at all concentrations of AYPGKF-NH$_2$ (Fig. 9D). Taken together, these data show that PAR4-mediated cell shape changes are independent of G$_{q/11}$, G$_{o}$, and G$_{q}$- mediated cell shape change is independent of G$_{q/11}$, G$_{o}$, and G$_{q}$-mediated cell shape change is independent of G$_{q/11}$, G$_{o}$, and G$_{q}$-

PAR4 Activation in Rat Primary Aortic Vascular Smooth Muscle Cells Leads to Cell Shape Changes. To examine whether PAR4 activation triggers cell blebbing in cells that endogenously express PAR4, we turned to the rat vascular smooth muscle cells. Vascular smooth muscle cells (VSMC) are reported to express PAR4 (Bretschneider et al., 2001; Dangwal et al., 2011), and in our hands, rat aortic smooth muscle cells from both Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats expressed PAR4 mRNA (Fig. 10I). We labeled the smooth muscle cell membrane with cell mask (ThermoFisher) and stimulated with AYPGKF-NH$_2$ (30 μM). Both WKY (Fig. 10A) and SHR (Fig. 10E) VSMC displayed cell shape changes resembling membrane blebs in response to PAR4 agonist treatment. To establish whether the signaling mechanism for these cell

![Figure 8](https://example.com/fig8.png)

**Fig. 8.** PAR4-mediated cell shape change is β-arrestin–dependent. Confocal micrographs of HEK-293 cells (A) or β-arrestin-1/2-KO HEK that were transiently transfected with 1 μg of PAR4-YFP (B) and were stimulated in AYPGKF-NH$_2$ (30 μM) for 2 minutes prior to confocal imaging. Arrows indicate bleb formation, and size bars are 20 μm. (C) Percentage of HEK-293 cells or β-arrestin-1/2-KO-HEK-293 transiently transfected with 1 μg of PAR4-YFP, displaying membrane blebbing in response to AYPGKF-NH$_2$ (30 μM) treatment. Data indicate mean ± S.D. of four independent experiments, and asterisks show significant differences; unpaired t test, *P* < 0.05. HEK-293 cells, transiently transfected with 1 μg of PAR4-YFP and 0.1 μg of either β-arrestin-1-Rluc or 0.1 μg of β-arrestin-2-Rluc were incubated in DMSO or YM254890 (100 nM) (D) for 20 minutes or PTX (100 ng/ml) for 18 hours (E) prior to testing in the BRET assay. Data represent mean ± S.D. of four independent experiments, and asterisks show significant differences; two-way ANOVA, Sidak’s multiple comparisons test, *P* < 0.05.
shape changes was the same in both VSMC and HEK-293 cells, we pretreated VSMC with blebbistatin or ROCK inhibitor (GSK269962) prior to activating PAR4 with AYPGKF-NH$_2$ (30 μM). In total, 67.25% ± 13.25% of WKY cells display cell shape changes in response to PAR4 agonist (Fig. 10, A and D), which was significantly reduced with blebbistatin treatment (9.0% ± 6.28%) (Fig. 10, B and D) or upon treatment with GSK269962 (6.5% ± 0.57%) (Fig. 10, C and D). Consistent with these findings, 65.96% ± 10.12% of SHR cells also displayed blebbing after PAR4 activation (Fig. 10E), and these responses were significantly reduced with blebbistatin (14.5% ± 3.7%) (Fig. 10, F and H) and with GSK269962 (15.7% ± 5.8%).

Fig. 9. PAR4-mediated cell shape change is G$_{bg}$-independent. Confocal micrographs of HEK-293 cells stably expressing PAR4-YFP were incubated in either DMSO (A) or gallein (10 μM) (B), a G$_{bg}$ inhibitor, for 20 minutes prior to AYPGKF-NH$_2$ treatment (30 μM) for 2 minutes and confocal imaging. Arrows show bleb formation, and size bars are 20 μm. (C) Percentage of cells stably expressing PAR4-YFP that exhibit membrane blebbing in response to AYPGKF-NH$_2$ (30 μM) in the presence of either DMSO (vehicle) or gallein (10 μM). Numbers above the bars indicate total number of cells scored for blebbing response. Data represent mean ± S.D. of four independent experiments, not significantly different by a Wilcoxon test. (D) HEK-293 cells, transiently transfected with 1 μg of PAR4-YFP and 0.1 μg of either -arrestin-1-Rluc or -arrestin-2-Rluc, were incubated in DMSO or with gallein (10 μM) for 20 minutes prior to testing in the BRET assay. Data represent mean ± S.D. of four independent experiments; not significantly different two-way ANOVA.

Fig. 10. PAR4 mediates membrane blebbing in vascular smooth muscle cells. Primary cultured vascular smooth muscle cells derived from WKY (A–D) or from SHR (E–H) rats were preincubated for 10 minutes in cell mask to stain the plasma membrane (shown in black); cells were then treated with AYPGKF-NH$_2$ (30 μM) and imaged by confocal microscopy. WKY cells and SHR cells were preincubated with DMSO (A and E) or with blebbistatin (10 μM) for 15 minutes (B and F) or with GSK269962 (100 nM) for 1 hour (C and G) prior to stimulation with AYPGKF-NH$_2$; arrows show bleb formation, and size bars are 20 μm. (D) Percentage of WKY cells displaying membrane blebbing in response to AYPGKF-NH$_2$ (30 μM) in the presence of either blebbistatin (10 μM) or GSK269962 (100 nM). Numbers above the bars indicate the total number of cells scored. Data indicate mean ± S.D. of four independent experiments, and asterisks show significant differences; Kruskal-Wallis test, Dunn’s multiple comparison test, $P < 0.05$. (H) Percentage of SHR cells displaying membrane blebbing in response to AYPGKF-NH$_2$ (30 μM) in the presence of either blebbistatin or GSK269962. Numbers above the bars indicate total number of cells scored. Data indicate mean ± S.D. of four independent experiments, and asterisks show significant differences; Kruskal-Wallis test, Dunn’s multiple comparison test, $P < 0.05$. (I) Gel image representing PCR product generated by use of PAR4-specific primers or β-actin–specific primers in WKY and SHR cells.
membrane blebbing occurs downstream of PAR4 activation and cellular responses that allow coagulation cascade enzymes immune response to injury and infection. PARs also elicit GPCRs is well established as critical regulators of the innate ration, and inflammation. In this context, the PAR family of an important regulator of wound healing, immune cell matu- and Grosse, 2008; Ikenouchi and Aoki, 2017). Blebbing is also embryonic development (Charras and Paluch, 2008; Fackler in invasion, cell locomotion, and regulation of cell polarity in leading to cellular responses including enhanced cell motility, such as thrombin and other serine proteinases to regulate various cellular functions. PAR1 and PAR4 serve as the receptors for thrombin on human platelets, although these receptors regulate different aspects of platelet activation (Coughlin, 1999; Kahn et al., 1999; Ma et al., 2005; Holinstat et al., 2006; Voss et al., 2007). PAR4 is described as the low-affinity thrombin receptor on human platelets. This lower affinity stems from the lack of a hirudin-like binding site for thrombin on PAR4 that is present on PAR1. In platelets, PAR4 activation typically requires a much higher concentration of thrombin to be present, and PAR4 activation typically results in more sustained calcium signaling compared with PAR1-dependent signaling (Covic et al., 2000; Shapiro et al., 2000).

Both PAR1 and PAR4 activation triggers platelet aggregation, with PAR4 signaling critical for full platelet spreading and formation of stable aggregates. In rodents, PAR4 serves as the sole thrombin receptor in platelets (Sambrano et al., 2001). It has been previously shown in Grq11 knockout mice that a thrombin-activated Grq11-mediated calcium response is neces- sary for platelet aggregation (Offermanns et al., 1997). How- ever, platelets from the mouse Grq11 knockout retained the ability to change shape in response to thrombin stimulation. Similar findings were reported in platelets treated with a small- molecule Grq11 antagonist, UBO-QIC, showing that platelet aggregation was inhibited without affecting shape change responses (Inamdar et al., 2015). Consistent with these findings, we have observed a cell shape change specific to PAR4 activation that is Grq11-independent and a RhoA-mediated phenomenon. Recent evidence suggests that PAR4 plays an important role in promoting platelet granule release and platelet-leukocyte interactions (Rigg et al., 2019), responses which also rely on a RhoA-mediated cytoskeletal rearrange- ment (Moers et al., 2003; Aslan and Mccarty, 2013). These findings raise the interesting possibility that different signal- ing pathways may underlie PAR4 regulation of distinct aspects of platelet activation, and further study is required to fully elucidate the role of different signaling cascades in

Discussion

We have demonstrated that activation of PAR4 with thrombin or the synthetic PAR4 activating peptide AYPGKF-NH2 causes a rapid cell shape change response in PAR4-transfected HEK-293 cells or in vascular smooth muscle cells that endogenously express PAR4. Cell shape changes are pharmacologically inhibited by blebbistatin and are consistent with membrane bleb formation. We observed membrane blebs that formed within 2–5 minutes of agonist treatment and lasted for up to 30 minutes. Membrane blebbing could be pharmacologically inhibited by the ROCK inhibitor GSK269962 or through CRISPR/Cas9-mediated knockout of RhoA. CRISPR/Cas9 knockout of β-arrestin-1 and 2 partially abolished PAR4-dependent membrane blebbing, as did overexpression of a dominant negative Grq12 protein. We further found PAR4-dependent membrane bleb formation to be independent of Go12, Gs, G13, and PKC (Fig. 11). Overall, our data suggest that Go12/RhoA-dependent membrane blebbing occurs downstream of PAR4 activation and β-arrestin recruitment.

Nonapoptotic cell membrane blebbing plays an important role in various physiologic and pathological processes. Various stimuli have been reported to trigger bleb formation, leading to cellular responses including enhanced cell motility, invasion, cell locomotion, and regulation of cell polarity in embryonic development (Charras and Paluch, 2008; Fackler and Grosse, 2008; Ikenouchi and Aoki, 2017). Blebbing is also an important regulator of wound healing, immune cell maturation, and inflammation. In this context, the PAR family of GPCRs is well established as critical regulators of the innate immune response to injury and infection. PARs also elicit cellular responses that allow coagulation cascade enzymes

![Fig. 11. PAR4 signaling pathways leading to membrane blebbing. Schema shows known PAR4 signaling partners that were interrogated pharmacologi- cally or genetically for their potential contribution to membrane blebbing. We find that agonist activation of PAR4 induces membrane blebbing that is independent of Grq11 signaling but is dependent on β-arrestin, Go12, and RhoA/ROCK. Pharmacological inhibitors that blocked PAR4-induced membrane blebbing are shown in red, and inhibitors that had no effect on membrane blebbing are shown in green. Biorender.com](image-url)
mediating PAR4 responses in platelets. Our studies suggest that in HEK-293 cells, the Gq11-coupled pathway and the RhoA pathway, which in this instance we show is downstream of Go12 coupling, can act independently and therefore may be independent targets for pharmacological manipulation.

PAR4 expression has also been reported in other cell types involved in the response to injury, including endothelial cells and smooth muscle cells (Bretschneider et al., 2001; Hamilton et al., 2001; Fujiwara et al., 2005; Ritchie et al., 2007). Here, we demonstrate that agonist stimulation of endogenous PAR4 in vascular smooth muscle cells leads to membrane blebbing.

This finding is consistent with a previous study that showed that another GPCR, Angiotensin II receptor type 1, mediates membrane blebbing by a RhoA-dependent mechanism in a vascular smooth muscle cell line (Godin and Ferguson, 2010). RhoA is well established as a mediator of changes in the plasma membrane actin cytoskeleton and in regulating GPCR-mediated cell shape change (Barnes et al., 2005; Godin and Ferguson, 2010; Aoki et al., 2016). Our findings add PAR4-dependent signaling to the cell surface signaling molecules that can trigger this pathway.

β-Arrestins are now well established as important molecular scaffolds linking GPCRs not only to molecular endocytic partners to facilitate receptor endocytosis but also to second messenger signal cascades (Ferguson, 2001; Magalhaes et al., 2012). For example, β-arrestins link GPCRs to p44/42 mitogen-activated protein kinase signaling from the endosome (Luttrell et al., 2001; Luttrell and Lefkowitz, 2002). PAR4 couples to both β-arrestin-1 and β-arrestin-2, and PAR4-mediated phosphorylation of AKT in platelets is β-arrestin-2-dependent (Li et al., 2011) activation. Recently, it has been shown that pepducin-mediated, β-arrestin–dependent activation of RhoAVROCK pathway mediated by β2-adrenergic receptor in cardiomyocytes is cardio-protective, highlighting the importance of β-arrestin signaling and also showing a link between β-arrestin and RhoA signaling molecules (Grisanti et al., 2018). A previous study has shown that β-arrestins are an integral part of focal adhesion dynamics and regulate clathrin and microtubule-dependent focal adhesion disassembly independently of GPCR signaling (Cleghorn et al., 2015). In our hands, there appears to be a role for β-arrestins in mediating membrane blebbing downstream of PAR4; however, additional GPCR-independent roles of β-arrestin on cytoskeletal dynamics cannot be ruled out. Our data here suggest that GPCR-mediated membrane blebbing and actin cytoskeletal rearrangements are predominantly G-protein–dependent and partially dependent on β-arrestins. Consistent with previous studies, we show that membrane blebbing is RhoA-dependent (Barnes et al., 2005; Godin and Ferguson, 2010); however, we find that PAR4 engages this signaling pathway through Go12 protein and not Gq11.

In conclusion, we have uncovered a PAR4-mediated cellular response that is independent of Gq11 coupling and occurs downstream of RhoA activation and β-arrestin signaling (Fig. 11). These data provide further evidence for pathway-selective signaling responses through PAR4 and may guide future development of PAR4 targeting strategies.