The Angiotensin II Type 1 Receptor Induces Membrane Blebbing by Coupling to Rho A, Rho Kinase, and Myosin Light Chain Kinase

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ABSTRACT

The angiotensin II type 1 receptor (AT1R) is a Gαq/11-coupled G protein-coupled receptor that is widely expressed in multiple tissues, including vascular smooth muscle cells, brain, and kidney. Activation of the AT1R in vascular smooth muscle cells leads to alterations in actin-based membrane protrusions such as lamellipodia, filopodia, and membrane blebs that ultimately lead to cell migration, which is important for the regulation of vascular tone. In the present study, we examine the role of small G proteins in mediating AT1R-induced alterations in membrane dynamics in human embryonic kidney 293 cells. We find that the activation of the AT1R with 100 nM angiotensin II results in the rapid formation of membrane blebs at early time points of agonist stimulation that cease within 40 min of agonist stimulation. AT1R-stimulated membrane bleb formation is independent of RalA, RalB, Rac1, cdc42, Arf6, and Ras, but it involves RhoA. Furthermore, membrane blebbing activated by the AT1R is attenuated in the presence of the β-arrestin amino-terminal domain, Ral GDP dissociation stimulator (RalGDS) β-arrestin binding domain, and short interfering RNA (siRNA) depletion of β-arrestin2. However, siRNA depletion of RalGDS protein did not affect membrane blebbing in response to AT1R activation. The inhibition of the downstream RhoA effectors Rho kinase (ROCK) and myosin light chain kinase (MLCK) effectively attenuated AT1R-mediated membrane blebbing. Thus, we show that membrane blebbing in response to AT1R signaling is dependent on β-arrestin2 and is mediated by a RhoA/ROCK/MLCK-dependent pathway.

Angiotensin II (AngII) is an octapeptide hormone and the active component of the renin-angiotensin system. It regulates blood pressure and volume, thirst, and sympathetic nervous activity, and it has a role in vascular remodeling in hypertension (Touyz and Schiffrin, 2000). The majority of the physiological effects of AngII are mediated through the angiotensin II type 1 receptor (AT1R), which is predominantly localized to vascular smooth muscle cells in the vasculature. AT1R-mediated intracellular signaling cascades transduce vascular effects such as contraction, cell growth, migration, extracellular matrix deposition, and inflammation (Touyz and Schiffrin, 2000; Touyz, 2005). AngII is critical for the maintenance of vessel wall structure and function and plays a major role in cardiovascular disease associated with vascular smooth muscle cell contraction and growth (Touyz and Schiffrin, 2000). Stimulation of the AT1R has been shown to lead to membrane ruffle formation (Cotton et al., 2007), stress fiber formation (Barnes et al., 2005), and cell migration (Hunton et al., 2005). AT1R-mediated changes in the membrane are important processes in vascular smooth muscle cell migration. This process underlies vascular remodeling in hypertension and atherosclerosis (Weir and Dzau, 1999).

Changes in the membrane such as ruffling and blebbing have been identified as important processes for cell migration. Mem-
brane ruffling and blebbing are distinct events that occur independent of cellular signaling cascades. Membrane blebbing is RhoA-, Rho kinase (ROCK)-, and myosin light chain kinase (MLCK)-dependent, and blebs are devoid of actin, mDia1, and Arp2/3 (Charras, 2008). In contrast, mDia1 and Arp2/3 localize to membrane ruffles, but ruffle formation is also RhoA-dependent (Chhabra and Higgs, 2007). In addition, Ral proteins have been shown to be involved in cell migration and reorganization of the actin cytoskeleton (Feig, 2003). Rals are small G proteins that cycle between an active GTP-bound state and an inactive GDP-bound state (Takei et al., 2001). Ral GDP dissociation inhibitor (RalGDS) was identified as a member of the cdc25 GDS family of proteins and was then found to be an effector of Ras (Albright et al., 1993; Hofer et al., 1994; Kikuchi and Williams, 1996). RalGDS is highly specific for RalA and RalB, whereby it facilitates the exchange of GDP for GTP on Rals (Albright et al., 1993; Matsubara et al., 1999; Wolthuis and Bos, 1999). RalA has been shown to be activated by GPCRs (Bhattacharya et al., 2002, Aziziye et al., 2009). It has been demonstrated previously that RalGDS forms a cytosolic complex with β-arrestin, and that in response to formyl-Met-Leu-Phε (fMLP) receptor stimulation, RalGDS is released from β-arr

ience and transfection experiments were performed in HEK293 cells. Cells were cultured in minimal essential medium supplemented with 8% fetal bovine serum (v/v) and 100 U/ml gentami

cells and were transfected using a modified calcium phosphate method with the amount of cDNA as indicated in the figure legends. Media were replaced 16 h after transfection. siRNAs were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

siRNA Transfection. The siRNAs used were the following: RalA, GGAUCACCGUGCAUUAGAU; RabB, CGCUCCAGUU-CAUGUAAGUA; RabGDS, CCAUCCUCGUUGGACCAs and mDia1, AUUUCUCUGCAUCUUAGGUA, all from Dharmaco. ROCK-1, MLCK, and β-arrestin siRNAs were ordered from QIA-GEN and included the following: β-arrestin 1, CTCGACCTTCT-GCAAGTGCTCA; β-arrestin 2, CTCGACCAAGATGACCGAGTTA; ROCK-1, CACAGTGAAATCATCCTTTA; and MLCK, CAG-CATCCATTGGAATGAAA. Scrambled siRNA (nontargeting siRNA 1) was purchased from Dharmaco. Experiments were performed 48 or 72 h after transfection, and knockdown of proteins was confirmed by Western blot.

Confocal Microscopy. Confocal microscopy was performed using a Zeiss LSM-510 laser scanning microscope equipped with a Zeiss 63x oil immersion lens (Carl Zeiss Inc., Thornwood, NJ). Live-cell imaging was performed on cells in untreated 35-mm glass-bottomed plates. Cells were kept at 37°C using a heated microscope stage. AT1R was labeled with rabbit anti-FLAG (Sigma-Aldrich)–conjugated Zenon Alexa Fluor 555 antibody (Invitrogen). Receptor was stimulated with the addition of 100 nM AngII and is necessary for AT1AR-induced stress fiber formation and actin polymerization (Lammers et al., 2008). Over
ing was done on cells that were treated with or without 100 nM AngII and then fixed and labeled with rabbit anti-FLAG 1:500 followed by}

Materials and Methods

Materials. siRNAs were purchased from Dharmaco RNA Technologies (Lafayette, CO) and from QIAGEN (Valencia, CA). Actin, MLCK, RabB, and ROCK-1 antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). RalA antibody was purchased from BD Biosciences Transduction Laboratories (Mississauga, ON), and mouse mDia1 antibody and horseradish peroxidase-conjugated anti-rabbit and anti-goat IgG secondary antibodies were purchased from GE Healthcare (Oakville, ON, Canada). Lipofectamine 2000 and Alexa Fluor 555 antibody labeling reagent, FM 4-64 dye, cytochalasin D, phalloidin-488, anti-rabbit 633, and GFP antibody were purchased from Invitrogen (Carlsbad, CA). Cells were pe}

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permeabilization and labeling with anti-rabbit 633 (1:1000) and phalloidin-488 (1:40).

**Immunoblotting.** Membranes were blocked with 10% milk in wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 0.05% Tween 20) for 1 h and then incubated with mouse anti-RalA (1:1000), goat anti-RalB (1:1000), rabbit anti-GFP (1:1000), mouse anti-ROCK (1:1500), and rabbit anti-MLCK (1:1500) antibodies in wash buffer containing 3% milk overnight. Membranes were rinsed three times with wash buffer and then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000), donkey anti-mouse IgG (1:2500), and rabbit anti-goat IgG (1:5000) in wash buffer containing 3% skim milk for 1 h. Membranes were rinsed three times with wash buffer and incubated with enhanced chemiluminescence Western blotting detection reagents.

**RhoA Activation Assay.** RhoA activation was assed in HEK293 using RhoA activation assay kit (Cell Biolabs, Inc., San Diego, CA). RhoA was visualized by Western blot using mouse anti-HA 1:500 followed by anti-mouse-horseradish peroxidase 1:1000 (GE Healthcare) and chemiluminescence using enhanced chemiluminescence Western blotting substrate (Pierce Chemical, Waltham, MA).

**Data Analysis.** Statistical significance was determined either by analysis of variance testing followed by post hoc multiple comparison testing or by paired t test using Prism software (GraphPad Software Inc., San Diego, CA).

### Results

**Stimulation of AT\textsubscript{1}R Induces Membrane Blebbing.** We have observed that the activation of the FLAG-AT\textsubscript{1}R with agonist resulted in rapid and robust changes in the cell shape, which could be described as plasma membrane blebbing. We found previously that the activation of the fMLP receptor in HEK293 cells resulted in plasma membrane blebbing. We found previously that the activation of cell shape, which could best be described as plasma membrane blebbing, we used a rat aortic vascular smooth muscle cell line (A10 cells) that expressed AT\textsubscript{1}R. To visualize A10 cell plasma membrane, cells were incubated in FM 4-64 dye and imaged at 2, 4, 12, and 20 min. Incubation in FM 4-64 dye did not result in membrane bleb formation (Fig. 1B). In contrast, A10 cells that were incubated in FM 4-64 dye and treated with 100 nM AngII displayed membrane blebs at 4, 12, and 20 min of agonist treatment (Fig. 1C). FM 4-64 dye was internalized by the cells over time, making imaging of later time points difficult. Because both ectopically and endogenously expressed AT\textsubscript{1}R induced membrane blebbing, we continued to study this process in HEK293 cells, which were much more readily manipulated.

Membrane bleb formation was shown previously to be initiated by the detachment of the plasma membrane from the actin cortex, which was generated by myosin II ATPase (Charras et al., 2005, 2006). HEK293 cells expressing FLAG-AT\textsubscript{1}R were fixed and stained to show both FLAG-AT\textsubscript{1}R at the plasma membrane and cellular actin (Fig. 2A). Cells treated with agonist displayed blebs, which exhibited staining for both FLAG and actin around the periphery of the bleb (Fig. 2B). However, the cores of the blebs were void of actin (Fig. 2B). This was consistent with previous characterization of membrane blebs (Charras et al., 2006). Blebbistatin, a myosin II ATPase inhibitor, was shown previously to function as an inhibitor of membrane blebbing (Charras et al., 2005). Zenon Alexa Fluor 555-labeled FLAG-AT\textsubscript{1}Rs were treated with 100 nM AngII for 15 min and scored for membrane blebbing responses. We found that blebbistatin treatment reduced the number of HEK293 cells that exhibited membrane blebbing from 65 ± 3 to 32 ± 2% (Fig. 2C). In contrast, the treatment of cells with cytochalasin D, an inhibitor of actin polymerization, had no effect on AT\textsubscript{1}R-dependent membrane blebbing (Fig. 2C). This was consistent with previous reports indicating that inhibition of actin polymerization by cytochalasin D did not attenuate membrane blebbing (Charras et al., 2005). Taken together, our results were consistent with
a previous study, which demonstrated that the membrane blebbing of RBL-2H3 cells was inhibited by blebbistatin but not cytochalasin D (Yanase et al., 2010).

Small GTPase Regulation of AT<sub>1</sub>R-Stimulated Membrane Blebbing. To examine the role of RalA and RalB in AT<sub>1</sub>R-stimulated membrane blebbing, HEK293 cells were transfected with siRNAs that specifically targeted the depletion of endogenous RalA and RalB proteins (Fig. 3A). However, the percentage of HEK293 cells exhibiting membrane blebbing in response to the activation of the AT<sub>1</sub>R was unchanged in cells lacking either RalA or RalB protein expression (Fig. 3B). To test the potential involvement of other small GTPases in the regulation AT<sub>1</sub>R-stimulated membrane blebbing, HEK293 cells were transfected with dominant-negative RhoA-T19N, Rac1-T17N, cdc42-T17N, Arf6-T27N, RalA-S28N, Ras-G15A, β-arr1N, or RalGDS 284 constructs, and blebbing was assessed. We found that in the presence of DN-RhoA-T19N, the number of cells exhibiting membrane blebbing in response to AT<sub>1</sub>R stimulation was reduced from 72 ± 5 to 10 ± 2% of cells (Fig. 3C). The overexpression of dominant-negative Rac1, cdc42, Arf6, RalA, and Ras mutants had no effect on AT<sub>1</sub>R-stimulated membrane blebbing (Fig. 3C). However, the overexpression of either the amino-terminal domain (amino acid residues 1–154) of β-arrestin 1 (β-arr1N) or the RalGDS β-arrestin binding domain (RalGDS 616–768) resulted in a significant attenuation of the number of cells that displayed membrane blebbing responses to 31 ± 3 and 20 ± 10%, respectively (Fig. 3C).

Effect of Kinase Inhibitors on AT<sub>1</sub>R-Stimulated Membrane Blebbing. Agonist stimulation of the AT<sub>1</sub>R results in rapid activation of a number of downstream protein kinases including protein kinase C (PKC), which might contribute to membrane blebbing responses. Therefore, we tested whether the inhibition of PKC, protein kinase A (PKA), protein kinase G, calmodulin (CaM) kinase II, and PI3 kinase might attenuate AT<sub>1</sub>R-stimulated membrane blebbing. The treatment of cells with the broad-spectrum PKC inhibitor staurosporine significantly reduced the number of HEK293 cells that displayed a membrane blebbing response to 3 ± 0.5% (Fig. 4A). However,
the treatment of HEK293 cells with either bisindolylmaleimide I (Bis I) or chelerythrine had no effect on AT1R membrane blebbing responses (Fig. 4A). Likewise, the inhibition of Ca2+-dependent PKC isoforms with either Go6976 or Go6983 had no effect on membrane blebbing (Fig. 4A). The inhibition of PKA (H-89), CaM kinase II (KT5823), and P13 kinase (wortmannin) also had no effect on AT1R-stimulated membrane blebbing (Fig. 4B). Thus, despite the effects of staurosporine on membrane blebbing, we concluded that AT1R-stimulated membrane blebbing was independent of the activation of PKA, PKC, CaM kinase II, and P13 kinase.

AT1R-Stimulated RalGDS Translocation and Rho Activity. Because we found that the activation of membrane blebbing by the AT1R was attenuated by the expression of either RhoA-T19N, RalGDS 616–768, or βArrest1N, we examined whether RhoA-T19A might attenuate the plasma membrane translocation of RalGDS in response to AT1R stimulation. In the absence of agonist, GFP-RalGDS was predominantly localized to the cytoplasm and displayed little membrane or nuclear localization in FLAG-AT1R-expressing HEK293 cells (Fig. 5A). However, similar to what we demonstrated previously for the fMLP receptor (Bhattacharya et al., 2002), in response to AngII treatment, GFP-RalGDS translocated from the cytoplasm to the plasma membrane (Fig. 5A). The overexpression of RhoA-T19N had no effect on the subcellular distribution of RalGDS in the absence of agonist treatment but prevented the plasma membrane translocation of GFP-RalGDS in response to AT1R activation (Fig. 5B). However, RhoA-T19N did not prevent AT1R-stimulated β-arrestin2 translocation (Fig. 5C). This suggested a functional interaction between RhoA and RalGDS. However, we were unable to demonstrate that RhoA could be coimmunoprecipitated with RalGDS (data not shown). Therefore, we examined whether overexpression of RalGDS might influence AT1R-stimulated GTP loading of RhoA. Similar to previous studies (Barnes et al., 2005), agonist-activation of AT1R resulted in a 2.4 ± 0.6-fold increase in GTP-bound RhoA precipitated with Rhotekin-RBD agarose (Fig. 5D). However, RalGDS expression had no effect on AT1R-stimulated activa-

**Fig. 5.** Translocation of RalGDS. A, representative live-cell confocal micrograph showing the subcellular distribution of GFP-RalGDS in HEK293 cells transiently transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT1R and 1 μg of peGFP-C2 plasmid cDNA encoding RalGDS before and after the treatment of cells with agonist (100 nM AngII) for 2 min. B, representative live-cell confocal micrograph showing the subcellular distribution of GFP-RalGDS in HEK293 cells transiently transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT1R and 1 μg of peGFP-C2 plasmid cDNA encoding RalGDS along with pcDNA3.1 plasmid cDNA encoding RhoA T19N before and after the treatment of cells with agonist (100 nM AngII) for 2 min. C, representative live-cell confocal micrograph showing the subcellular distribution of β-arrestin2-GFP in HEK293 cells transiently transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT1R and 1 μg of peGFP-C2 plasmid cDNA encoding RalGDS along with pcDNA3.1 plasmid cDNA encoding RhoA T19N before and after the treatment of cells with agonist (100 nM AngII) for 2 min. D, representative immunoblot showing activated GTP-bound RhoA precipitated with Rhotekin-RBD agarose from HEK293 cells. HEK293 cells were transiently transfected with 5 μg of pcDNA3.1 encoding FLAG-AT1R and with 3 μg of pcDNA3.1 encoding HA-RhoA along with either 1 μg of empty peGFP-C1 plasmid cDNA or 1 μg of peGFP-C1 plasmid cDNA encoding RalGDS in the absence and presence of agonist treatment (100 nM AngII) for 2 min. Graph shows the mean ± S.E.M. of six independent experiments. Asterisks indicate significant differences compared with control (GFP-transfected and untreated) (**p < 0.05**).
tation of RhoA (Fig. 5D). Thus, these results indicated that RalGDS might potentially function as an effector of RhoA but does not regulate RhoA activity.

**Role of β-Arrestin and RalGDS in AT1, R-Stimulated Membrane Blebbing.** Because the expression of either RalGDS 616–768 or the β-arrestin amino-terminal domain reduced AT1, R-stimulated membrane blebbing and RhoA-T19N prevented GFP-RalGDS membrane translocation, we explored whether the depletion of endogenous RalGDS and β-arrestin protein would alter membrane blebbing responses. When tested, the treatment of cells with a RalGDS-specific siRNA had no effect on the number cells that scored positive for a membrane blebbing response to AT1, R activation (Fig. 6A). However, we were unable to detect endogenous RalGDS expression in the cells. The ability of the RalGDS-specific siRNA to deplete RalGDS expression was confirmed in cells transfected with GFP-RalGDS (Fig. 6B). The treatment of HEK293 cells with a β-arrestin1-specific siRNA had no effect on AT1, R-stimulated membrane blebbing, whereas the treatment of cells with a β-arrestin2-specific siRNAs significantly reduced the number of cells exhibiting membrane blebbing responses (Fig. 6C). Treatment of cells with both β-arrestin1 and β-arrestin2 siRNA did not effectively reduce the number of cells displaying membrane blebbing (Fig. 6C), indicating that this effect was specific for β-arrestin2 and was potentially antagonized by β-arrestin1. Both the β-arrestin1 and β-arrestin2 siRNAs effectively reduced endogenous β-arrestin1 (top band) and β-arrestin2 (bottom band) expression (Fig. 6D). Thus, whereas Rho-dependent membrane blebbing in response to AT1, R activation was β-arrestin 2-dependent, it seemed to be independent of RalGDS.

**ROCK and MLCK Mediate RhoA-Dependent Membrane Blebbing.** Because we found that AT1, R-mediated membrane blebbing was dependent on RhoA, we examined which downstream RhoA effector proteins were involved in AT1, R-stimulated membrane blebbing responses. To examine the role of mDia1 in the regulation of AT1, R membrane blebbing, we used both dominant-negative protein expression and siRNA knockout approaches. The siRNA treatment of HEK293 cells to knockdown endogenous mDia1 resulted in a reduction of mDia1 protein expression (Fig. 7A) but had no effect on membrane blebbing responses to AT1, R activation (Fig. 7B). Likewise, the overexpression of a dominant-negative mDia1 had no effect on AT1, R-stimulated membrane blebbing (Fig. 7B). In contrast, the treatment of cells with the MLCK inhibitor ML-9 and the ROCK inhibitor Y-27632 reduced the fraction of HEK293 cells exhibiting membrane blebbing responses to 22 ± 5 and 20 ± 5%, respectively, compared with 67 ± 4% in cells treated with DMSO alone (Fig. 7C). Treatment of cells with either ROCK-specific or MLCK-specific siRNAs resulted in the depletion of either ROCK protein or MLCK proteins, respectively (Fig. 7D). The treatment of HEK293 cells with MLCK- or ROCK-targeted siRNA also resulted in a significant decrease in membrane blebbing from 69 ± 3 to 39 ± 6 and 34 ± 9%, respectively (Fig. 7E). These results indicated that both ROCK and MLCK functioned as RhoA effector proteins underlying membrane blebbing in response to AT1, R activation.

**Discussion**

In the present study, we found that the activation of the AT1, R results in a rapid and sustained alteration of

![Fig. 6](https://example.com/fig6.png)

**Fig. 6.** Cells depleted for β-arrestin2 but not RalGDS show a reduction in membrane blebbing. A, membrane blebbing in response to 100 nM AngII treatment of HEK293 cells transiently transfected with 5 μg of pcDNA3.1 encoding FLAG-AT1 and 100 pmol scramble (scr) siRNA or RalGDS-specific siRNA. Data represent the mean ± S.E.M. of four independent experiments, and the numbers above the columns indicate the number of cells scored for blebbing responses. B, shown is a representative autoradiograph of GFP-RalGDS siRNA depletion of cells transfected with 5 μg of pcDNA3.1 cDNA plasmid encoding GFP-RalGDS along with 100 pmol of either scrambled or RalGDS siRNA. C, membrane blebbing in response to 100 nM AngII treatment of HEK293 cells transiently transfected with 5 μg of pcDNA3.1 encoding FLAG-AT1 and 100 pmol of either scrambled, β-arrestin1, β-arrestin2, or both β-arrestin1 and β-arrestin2 siRNAs. Data represent the mean ± S.E.M. of six independent experiments, and the numbers above the columns indicate the number of cells scored. Asterisks indicate significant differences compared with control (p < 0.05). D, a representative autoradiograph of siRNA-mediated depletion of endogenous β-arrestin1 and β-arrestin2 protein in cells transfected with 5 μg of pcDNA3.1 cDNA plasmid encoding FLAG-AT1 and treated with 100 pmol scrambled, β-arrestin1, β-arrestin2, or both β-arrestin1 and β-arrestin2 siRNAs. IB, immunoblot.
HEK293 and A10 cell shape that is best described as membrane blebbing. This membrane blebbing response requires β-arrestin2 and RhoA protein expression and could be blocked by using inhibitors of either ROCK or MLCK. In addition, AT1R-mediated membrane blebbing is independent of PKA, PKC, and CaM kinase II activation. Moreover, AT1R-stimulated changes in cell shape do not involve a RalGDS/Ral-mediated pathway. The AT1R-stimulated changes in HEK293 cell shape are observed within 3 min of agonist stimulation and persist for up to 40 min, at which time the blebbing ceases and the cells return to their original shape. The membrane blebbing induced in response to AT1R-stimulated membrane blebbing resembles alterations in cell shape induced by the activation of the neurokinin receptor (Meshki et al., 2009). Similar to what we report here, neurokinin receptor-mediated blebbing is mediated by a RhoA-, ROCK-, and MLCK-dependent pathway. These membrane blebs seem to be distinct from RhoA-mediated membrane ruffling, which involves the activation of mDia1 and Arp2/3 (Chhabra and Higgs, 2007; Charras, 2008).

We demonstrated previously that fMLP-mediated changes in cell shape involved a β-arrestin/RalGDS/Rala-mediated pathway. However, although the activation of the AT1R results in RalGDS translocation to the plasma membrane, we found that AT1R-induced membrane blebbing does not require RalGDS, Rala, or RalB protein expression. The activation of the AT1R can also induce membrane ruffling, which has been demonstrated to involve the activation of ARF6 and Rac1 (Cotton et al., 2007). In the present study, the AT1R-induced alterations in cell shape that resemble membrane blebs are independent of either Arf6 or Rac1 activity but rather require the RhoA-dependent activation of ROCK and MLCK. This indicates that the activation of membrane ruffling and membrane blebbing by the AT1R involves the activation of distinct downstream signaling pathways.

We demonstrated previously that β-arrestins form a complex with RalGDS in resting cells and that after agonist stimulation, this complex dissociates, and both β-arrestin and RalGDS protein translocate to the plasma membrane, leading to the activation of Rala (Bhattacharya et al., 2002). We have shown here that AT1R-mediated membrane blebbing is independent of Rala but is β-arrestin2-dependent and can be inhibited by the amino-terminal domain of either β-arrestin1 or the RalGDS β-arrestin-binding domain. This suggests that both β-arrestin and RalGDS might contribute to the regulation of RhoA-mediated membrane blebbing. Consistent with this, we found that the depletion of β-arrestin2 by siRNA inhibits AT1R-stimulated membrane blebbing. In contrast, RalGDS depletion by siRNA has no effect on AT1R-stimulated membrane blebbing. β-Arrestin1 has been shown previously to activate RhoA-dependent stress fiber formation in response to AT1R, which suggests that RhoA may be an effector of β-arrestin-dependent cell signaling (Barnes et al., 2005). However, the role of RalGDS in the regulation of RhoA-dependent membrane blebbing in response to AT1R is less clear. Expression of RalGDS 616–768 blocks membrane blebbing, but the depletion of RalGDS protein expression has no effect. Thus, it is possible that effect of
RalGDS 616–768 on membrane blebbing is independent of RhoA signaling and that this mutant blocks membrane blebbing by sequestering β-arrestin in the cytosol. However, a role for RalGDS in the regulation of RhoA signaling cannot be entirely discounted, because RhoA-T19N prevents RalGDS translocation to the plasma membrane. Nevertheless, we are unable to demonstrate that RalGDS contributes to the activation of RhoA.

Agonist stimulation of AT1R results in Ga131-dependent activation of phospholipase Cβ, leading to increases in intracellular diacylglycerol and inositol 1,4,5-trisphosphate formation, which stimulates the release of calcium from intracellular stores and promotes the activation of PKC. We have demonstrated previously that PKC translocates to the plasma membrane within seconds of agonist activation and thus can be expected to precede the formation of membrane protrusions (blebs) that occur in response to AT1R stimulation (Polichia et al., 2006). However, the treatment of HEK293 cells with a variety of PKC inhibitors failed to block AT1R-stimulated membrane blebbing, indicating that membrane blebbing is independent of PKC activity. This observation is similar to what is observed for neurokinin receptor-mediated membrane blebbing, which is independent of phospholipase C activity (Meskhi et al., 2009). Likewise, PKA activity did not contribute to membrane blebbing. However, although Bis I, chelerythrine, Go6976, and Go6983 failed to block AT1R-stimulated membrane blebbing, the treatment of HEK293 cells with staurosporine led to a significant attenuation of membrane blebbing. The mechanism by which staurosporine prevents membrane blebbing is unclear. The main biological activity of staurosporine is to competitively inhibit ATP binding to protein kinases, and it binds to many protein kinases with high affinity but with little selectivity (Karaman et al., 2008). Thus, it is possible that staurosporine may antagonize either ROCK or MLCK kinase activity. Membrane blebbing in response to thrombin receptor activation has also been associated with phosphoinositide 3-kinase (Vemuri et al., 1996).

Rho GTPases mediate alterations in plasma membrane dynamics via an interaction with a variety of downstream regulatory proteins, including mDia1. RhoA activates mDia1 catalytic activity by binding to the amino-terminal domain of mDia1, which releases the autoinhibitory carboxyl-terminal domain of mDia1 (Lammers et al., 2008). Previous studies have shown that mDia1 is localized to membrane ruffles and is required for RhoA-mediated neutrophil chemotaxis (Charras, 2008; Shi et al., 2009). However, we found that AT1R-stimulated membrane blebbing responses are not inhibited by either the expression of a dominant-negative mutant of mDia1 or siRNA-mediated depletion of mDia1 protein expression. However, our work does not rule out the possibility that our experimental manipulations did not either sufficiently deplete mDia1 protein expression or attenuate endogenous mDia1 protein function to an extent required to observe attenuated membrane blebbing. Thus, we cannot conclusively rule out a role for mDia1 function in AT1R-stimulated membrane blebbing responses. However, Rho-dependent activation of the actin-myosin machinery also involves the phosphorylation of myosin light chain by ROCK and MLCK. We show here that ROCK and MLCK activity are required for the formation of membrane blebs in response to the activation of the AT1R. This is consistent with previous studies with the neurokinin 1 receptor and the observation that constitutively active ROCK induces bleb formation in Walker carcinosarcoma cells, which is inhibited by the MLCK inhibitor ML-7 (Gutjahr et al., 2005; Meskhi et al., 2009).

Membrane blebbing is less well studied than other actin-based membrane protrusions such as lamellipodia or filopodia and has been commonly associated with apoptotic response (Charras, 2008). Membrane blebs can be described as spherical plasma membrane protrusions and can be associated with normal physiological responses induced by receptor activation such as cell movement, cytokinesis, and cell spreading (Charras, 2008). Blebbing also results in lamellipodia-independent migration of invasive tumor cells and stem cells (Charras and Paluch, 2008). Cell migration induced by the activation of AT1R expressed in vascular smooth muscle cells represents an important mechanism involved in vascular remodeling associated with hypertension and cardiovascular disease (Touyz and Schiffrin, 2000). Rhoa/ROCK signaling plays an important role in both normal physiology vascular function and the regulation of vascular remodeling associated with cardiovascular disease (Calò and Pessina, 2007). Therefore, characterizing the molecular mechanisms by which the AT1R regulates the actin cytoskeleton and plasma membrane dynamics is essential for understanding both the normal physiological and pathophysiological signals mediated by this receptor. In summary, we provide evidence that agonist stimulation of AT1R leads to plasma membrane blebbing responses by activation of RhoA and subsequent coupling to the ROCK/MLCK pathway.

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