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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 (AT_1R) is a $G\alpha_{q/11}$-coupled G protein-coupled receptor that is widely expressed in multiple tissues, including vascular smooth muscle cells, brain, and kidney. Activation of the AT_1R 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 AT_1R-induced alterations in membrane dynamics in human embryonic kidney 293 cells. We find that the activation of the AT_1R 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. AT_1R-stimulated membrane bleb formation is independent of RalA, RalB, Rac1, cdc42, Arf6, and Ras, but it involves RhoA. Furthermore, membrane blebbing activated by the AT_1R 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 AT_1R activation. The inhibition of the downstream RhoA effectors Rho kinase (ROCK) and myosin light chain kinase (MLCK) effectively attenuated AT_1R-mediated membrane blebbing. Thus, we show that membrane blebbing in response to AT_1R 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 (AT_1R), which is predominantly localized to vascular smooth muscle cells in the vasculature. AT_1R-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 AT_1R 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). AT_1R-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-

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ABBREVIATIONS: AngII, angiotensin II; AT_1R, angiotensin II type 1 receptor; Bis I, bisindolylmaleimide I; Ca^{2+}, calcium; CaM, calmodulin; fMLP, formyl-Met-Leu-Phe; GFP, green fluorescent protein; HEK, human embryonic kidney; MLCK, myosin light chain kinase; PKA, protein kinase A; PKC, protein kinase C; RalGDS, Ral GDP dissociation stimulator; ROCK, Rho kinase; siRNA, short interfering RNA; DMSO, dimethyl sulfoxide; HA, hemagglutinin; PI3 kinase, phosphatidylinositol 3-kinase; H-89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; KT5823, 2,3,9,10,11,12-hexahydro-10*R*-methoxy-2,9-dimethyl-1-oxo-9*S*,12*R*-epoxy-1H-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid, methyl ester; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; ML-9, 1- [(5-chloro-1-naphthalenyl)sulfonyl]hexahydro-1H-1,4-diazepine, monohydrochloride; Y-27632, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride; Gö9683, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide.

brane ruffling and blebbing are distinct events that occur through independent 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 (Takai et al., 2001). Ral GDP dissociation stimulator (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, Aziziyeh 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-Phe (fMLP) receptor stimulation, RalGDS is released from β-arrestin and translocates to the plasma membrane. fMLP receptor-stimulated membrane ruffling is blocked by a mutant RalGDS (amino acids 616–768), which encodes the β-arrestin binding region of RalGDS and by the expression of the amino-terminal domain of β-arrestin (Bhattacharya et al., 2002).

RhoA is an additional small G protein that is implicated in regulating plasma membrane dynamics. RhoA is activated by AngII and is necessary for $AT_{1A}R$-induced stress fiber formation in human embryonic kidney (HEK) 293 cells (Barnes et al., 2005). Activated RhoA is localized to membrane ruffles (Kurokawa et al., 2005). The proteins ROCK and mDia1 are both RhoA effectors and have been shown to play a role in changes in actin cytoskeletal reorganization. RhoA acts through ROCK to form stress fibers (Barnes et al., 2005), and ROCK has been shown to be involved in arterial contraction induced by AngII (Matrougui et al., 2001). RhoA has also been found to be localized and active at the leading edge of migrating cells. Dominant-negative RhoA expression blocks membrane ruffling in Cos1 cells stimulated with epidermal growth factor in a ROCK-independent but mDia1-dependent manner (Kurokawa et al., 2005). mDia1 is a member of the ubiquitous formin protein family. These proteins are activated by interaction with Rho GTPases and are then able to mediate actin polymerization (Lammers et al., 2008). Overexpression of the GTP-Rho binding domain of mDia1 causes spontaneous membrane ruffles (Krebs et al., 2001).

In the present study, we tested the hypothesis that AT_1R-induced membrane blebbing was RalA-dependent, similar to what was described previously for the fMLP receptor (Bhattacharya et al., 2002). We find that AT_1R-stimulated membrane blebbing in HEK293 cells is independent of RalA and is mediated by RhoA-dependent activation of ROCK and MLCK.

Materials and Methods

Materials. siRNAs were purchased from Dharmacon RNA Technologies (Lafayette, CO) and from QIAGEN (Valencia, CA). Actin, MLCK, RalB, 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 preincubated with bisindolymaleimide 1, staurosporine, chelerythrine chloride, *N*-[2-(4-bromocinnamylamino) ethyl]-5-isoquinoline (H-89), wortmannin, 2,3,9,10,11,12-hexahydro-10*R*-methoxy-2,9-dimethyl-1-oxo-9*S*,12*R*-epoxy-1H-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid, methyl ester (KT5823), autocamtide-2 inhibitory-related peptide, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole (Gö6976), blebbistatin, or 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide (Gö6983) and were purchased from EMD Chemicals (Gibbstown, NJ). ML-9 was purchased from Tocris Cookson Inc. (Ellisville, MO), and (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride (Y-27632) was purchased from Cayman Chemical (Ann Arbor, MI). Rabbit anti-FLAG antibody, mouse anti-HA antibody, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Dr. John Copeland (University of Ottawa, Ottawa, ON, Canada) provided the mDia1 plasmid.

Cell Culture and Transfection. Experiments were performed in HEK293 cells. Cells were cultured in minimal essential media supplemented with 8% fetal bovine serum (v/v) and 100 μg/ml gentamicin. All reagents were obtained from Invitrogen. Cells were seeded in 100-mm culture dishes 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 Transcfection. The siRNAs used were the following: RalA, GGACUACGCUGCAAUUAGAUU; RalB, CGCUUCAGUUCAUGUAUGAUU; RalGDS, CCAUCUUCCUGUGUACCUA; and mDia1, AUUCUUCUGCAUCAUAUGGUU, all from Dharmacon. ROCK-1, MLCK, and β-arrestin siRNAs were ordered from QIAGEN and included the following: β-arrestin 1, CTCGACGTTCTGCAAGGTCTA; β-arrestin 2, CTCGAACAAGATGACCAGGTA; ROCK-1, CAAGCTCGAATTACATCTTTA; and MLCK, CAGCATCCATGGCTAATGAAA. Scrambled siRNA (nontargeting siRNA 1) was purchased from Dharmacon. 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 63× 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. AT_1R 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 (final concentration). Visualization of antibody-labeled receptor with GFP-RalGDS was performed by dual excitation (488/543 nm) and emission band pass from 505 to 530 (GFP) and long pass at 560 (Alexa Fluor 555) filter sets. Cells were preincubated with either 1 μM bisindolymaleimide 1, 1 μM staurosporine, 5 μM chelerythrine chloride, 100 nM H-89, 200 nM wortmannin, 500 nM KT5823, 100 nM autocamtide-2 inhibitory-related peptide, 100 nM Gö6976, 100 nM Gö9683, 50 μM ML-9, 10 μM Y-27632, 5 μM, blebbistatin, or 2.5 μM cytochalasin D. HEK293 cells were then imaged in the presence of the inhibitors treated with and without 100 nM AngII. Cells that were expressing receptor were counted and scored for blebbing or nonblebbing. Percentage of blebbing was calculated as the percentage of cells exhibiting membrane blebbing responses to AngII treatment. Actin imaging 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

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-HA (1:500), rabbit anti-actin (1:1000), rabbit anti-ROCK (1:500), and rabbit anti-MLCK (1:500) 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$_1$R Induces Membrane Blebbing. We have observed that the activation of the FLAG-AT$_1$R with agonist resulted in rapid and robust changes in the cell shape, which could best be described as plasma membrane blebbing. We found previously that the activation of the fMLP receptor in HEK293 cells resulted in β-arrestin/RalGDS/RalA-dependent cytoskeletal reorganization (Bhattacharya et al., 2002). Therefore, we examined whether AT$_1$R-mediated membrane blebbing involved a similar mechanism. In HEK293 cells transiently transfected to express FLAG-AT$_1$R, FLAG-AT$_1$R was uniformly distributed at plasma membrane (Fig. 1A) and in response to agonist treatment with 100 nM AngII, extensive membrane blebbing of the HEK293 cells was observed within 2 to 3 min and persisted for up to 40 min in the continued presence of agonist (Fig. 1A). To determine whether the activation of endogenously expressed AT$_1$R resulted in membrane blebbing, we used a rat aortic vascular sooth muscle cell line (A10 cells) that expressed AT$_1$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$_1$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. This detachment required contraction of the actin cortex, which was generated by myosin II ATPase (Charras et al., 2005, 2006). HEK293 cells expressing FLAG-AT$_1$R were fixed and stained to show both FLAG-AT$_1$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$_1$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$_1$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

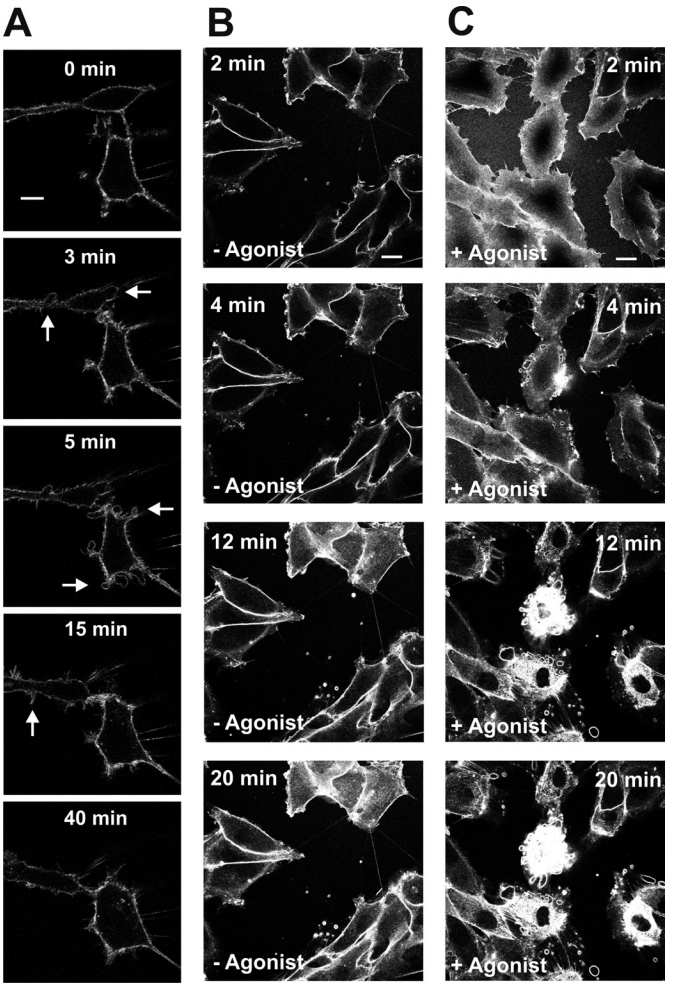

Fig. 1. AT$_1$R-induced membrane blebbing. A, representative confocal micrographs of HEK293 cells transiently transfected with 5 μg of pcDNA3.1 encoding FLAG-AT$_1$R and labeled with rabbit anti-FLAG antibody conjugated to Zenon Alexa Fluor 555 to stain cell surface receptors, in the absence of agonist, and after 3-, 5-, 15-, and 40-min treatment with 100 nM AngII. Arrows, blebs. B, A10 cells treated with 5 μg/ml FM 4-64 dye for 2, 4, 12 and 20 min. C, A10 cells treated with 5 μg/ml FM 4-64 dye and 100 nM AngII for 2, 4, 12, and 20 min. Scale bars, 10 μm; images are representative of three independent experiments.

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_1R-Stimulated Membrane Blebbing. To examine the role of RalA and RalB in AT_1R-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_1R 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_1R-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_1R 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_1R-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_1R-Stimulated Membrane Blebbing. Agonist stimulation of the AT_1R 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_1R-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,

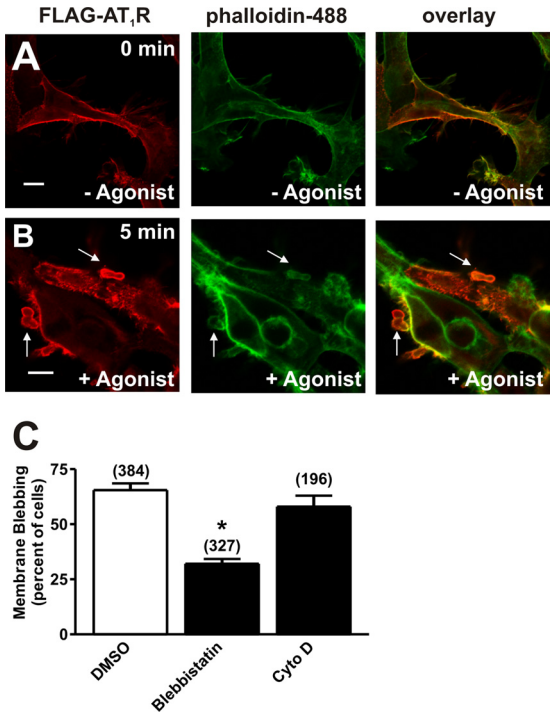

Fig. 2. Blebbing is actin-independent. A, confocal micrograph showing HEK293 cells transfect with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R that have been labeled with rabbit anti-FLAG (red) and phalloidin-488 (green). B, cells have been treated with 100 nM AngII for 5 min; arrows, blebs. Scale bars, 10 μm; images are representative of three independent experiments. C, percentage of HEK293 cells transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R that exhibit membrane blebbing responses in response to treatment with 100 nM AngII in the presence of either DMSO (control), 5 μM blebbistatin, or 2.5 μM cytochalasin D (Cyto D). Numbers above bars indicate the number of cells that were scored for blebbing responses. Data represent the mean ± S.E.M. of four independent experiments. Asterisks indicate significant differences compared with control ($p < 0.05$).

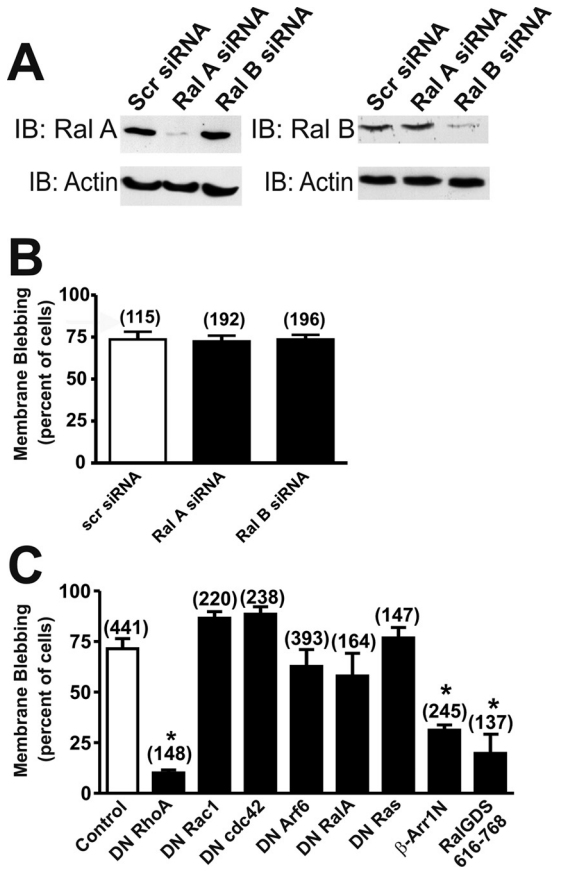

Fig. 3. Small GTPase regulation of AT_1R-induced membrane blebbing. A, Western blot analysis showing siRNA depletion of RalA or RalB proteins in HEK293 cells. B, graph showing the percentage of HEK293 cells transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R that exhibit membrane blebbing responses in response to 100 nM AngII when cotransfected with 100 pmol of either scrambled siRNA, RalA siRNA, or RalB siRNA. Numbers above bars indicate the number of cells that were scored for blebbing responses. Data represent the mean ± S.E.M. of four independent experiments. C, graph showing the percentage of HEK293 cells transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R that exhibit membrane blebbing responses in response to 100 nM AngII when cotransfected with DN RhoA, DN Rac1, DN cdc43, DN Arf6, DN RalA, DN Ras, β-Arr1N, and RalGDS 616–768. Cells were cotransfected with 1 μg of either peGFP-C2 encoding dominant-negative RhoA-T19N, Rac1-T17N, cdc42-T17N, Arf6-T27N, RalA-S28N, Ras-G15A, RalGDS 616–768, or peGFP-N1 encoding β-Arr1N. Cells were treated with 100 nM AngII for 2 min before imaging and were scored for membrane blebbing responses. Numbers above bars indicate the number of cells that were scored for blebbing responses. Data represent the mean ± S.E.M. of four independent experiments. Asterisks indicate significant differences compared with control ($p < 0.05$).

the treatment of HEK293 cells with either bisindolylmaleimide I (Bis I) or chelerythrine had no effect on AT_1R membrane blebbing responses (Fig. 4A). Likewise, the inhibition of Ca^{2+}-dependent PKC isoforms with either Gö6976 or Gö6983 had no effect on membrane blebbing (Fig. 4A). The inhibition of PKA (H-89), CaM kinase II (KT5823), and PI3 kinase (wortmannin) also had no effect on AT_1R-stimulated membrane blebbing (Fig. 4B). Thus, despite the effects of staurosporine on membrane blebbing, we concluded that AT_1R-stimulated membrane blebbing was independent of the activation of PKA, PKC, CaM kinase II, and PI3 kinase.

AT_1R-Stimulated RalGDS Translocation and Rho Activity. Because we found that the activation of membrane blebbing by the AT_1R was attenuated by the expression of either RhoA-T19N, RalGDS 616–768, or βArr1N, we examined whether RhoA-T19A might attenuate the plasma membrane translocation of RalGDS in response to AT_1R stimulation. In the absence of agonist, GFP-RalGDS was predominantly localized to the cytoplasm and displayed little membrane or nuclear localization in FLAG-AT_1R-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 AT_1R activation (Fig. 5B). However, RhoA-T19N did not prevent AT_1R-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 AT_1R-stimulated GTP loading of RhoA. Similar to previous studies (Barnes et al., 2005), agonist-activation of AT_1R 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 AT_1R-stimulated activa-

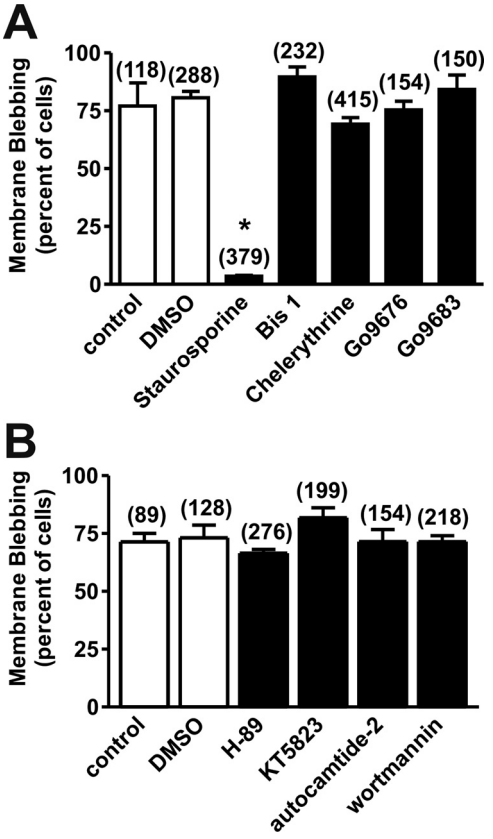

Fig. 4. Effect of PKC inhibition on AT_1R-induced membrane blebbing. A, graph showing the percentage of HEK293 cells transfected with 5 μg of pcDNA3.1 plasmid encoding FLAG-AT_1R exhibiting membrane blebbing responses in response to 100 nM AngII treatment. HEK293 cells were treated with either Hanks' balanced salt solution (control), DMSO, staurosporine (1 μM), Bis I (1 μM), chelerythrine (5 μM), Gö6976 (100 nM), or 100 nM Gö9683 (100 nM) for 10 min before agonist treatment for 2 min. Data represent the mean ± S.E.M. of four to six independent experiments. Asterisks indicate significant differences compared with control ($p < 0.05$). B, graph shows the percentage of HEK293 cells transfected with 5 μg of pcDNA3.1 plasmid encoding FLAG-AT_1R exhibiting membrane blebbing responses in response to 100 nM AngII treatment. HEK293 cells were treated with Hanks' balanced salt solution (control), DMSO, H-89 (100 nM), KT5823 (500 nM), autocamtide-2 (100 nM), or wortmannin (200 nM) for 10 min before agonist treatment for 2 min. Data represent the mean ± S.E.M. of three to four independent experiments. Numbers above the bars indicate the number of cells scored.

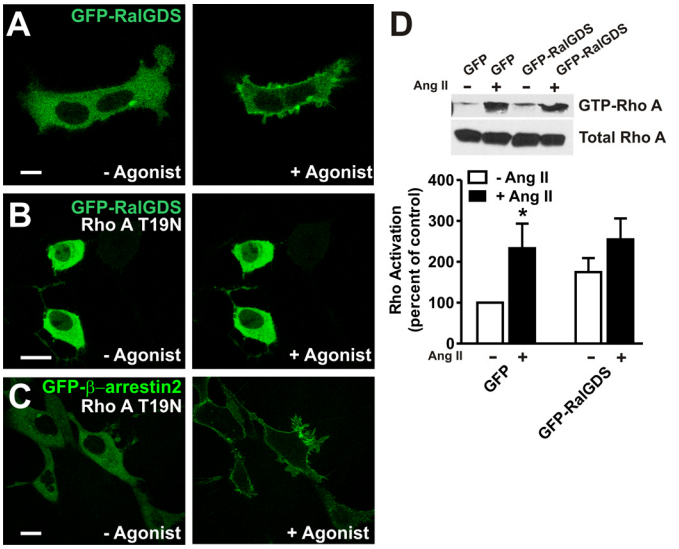

Fig. 5. Translocation of RalGDS. A, representative live-cell confocal micrograph showing the subcellular distribution of GFP-RalGDS in HEK293 cells transiently transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and 1 μg of peGFP-C2 plasmid cDNA encoding RalGDS before and after the treatment 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-AT_1R 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-AT_1R and 1 μg of peGFP-N1 plasmid cDNA encoding β-arrestin2 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 3 μg of pcDNA3.1 encoding FLAG-AT_1R 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) ($p < 0.05$).

tion 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 AT_1R-Stimulated Membrane Blebbing. Because the expression of either RalGDS 616–768 or the β-arrestin amino-terminal domain reduced AT_1R-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 AT_1R 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 AT_1R-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 AT_1R 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 AT_1R-mediated membrane blebbing was dependent on RhoA, we examined which downstream RhoA effector proteins were involved in AT_1R-stimulated membrane blebbing responses. To examine the role of mDia1 in the regulation of AT_1R membrane blebbing, we used both dominant-negative protein expression and siRNA knockdown 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 AT_1R activation (Fig. 7B). Likewise, the overexpression of a dominant-negative mDia1 had no effect on AT_1R-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 siRNA 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 AT_1R activation.

Discussion

In the present study, we found that the activation of the AT_1R results in a rapid and sustained alteration of

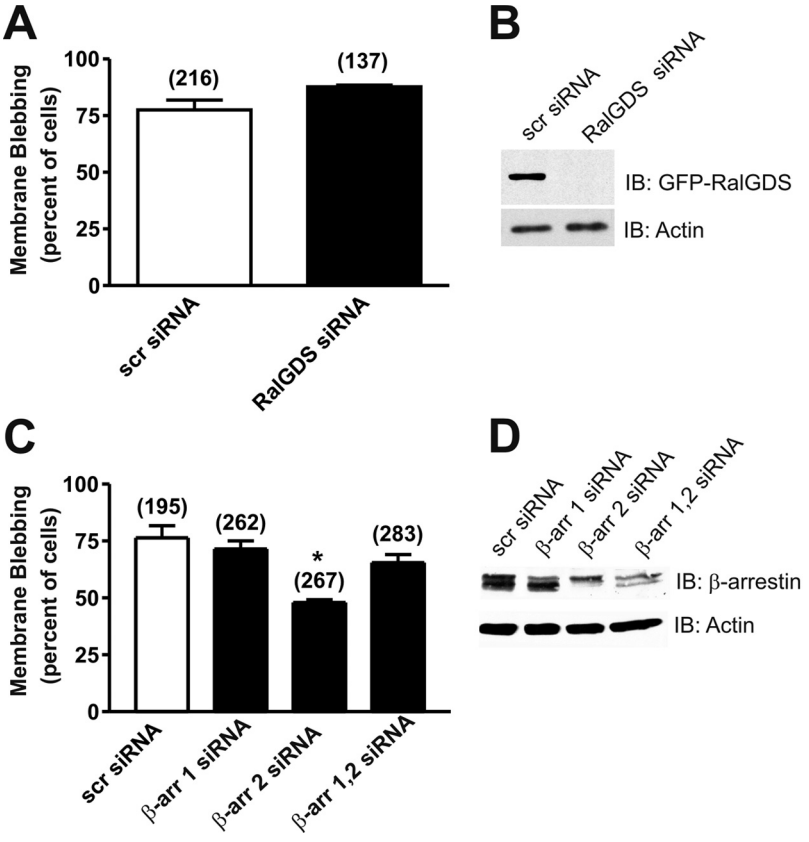

Fig. 6. Cells depleted for β-arrestin2 but not RalGDS show a reduction in membrane blebbing. A, membrane blebbing in response to 100 nM AngII treatment of HEK293 cells transiently transfected with 5 μg of pcDNA3.1 encoding FLAG-AT_1R and 100 pmol scramble (scr) siRNA or RalGDS-specific siRNA. Data represent 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 FLAG-AT_1R and 1 μg of pEGFP-C2 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-AT_1R 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-AT_1R 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, AT_1R-mediated membrane blebbing is independent of PKA, PKC, and CaM kinase II activation. Moreover, AT_1R-stimulated changes in cell shape do not involve a RalGDS/Ral-mediated pathway. The AT_1R-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 AT_1R-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 Rho A-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 AT_1R results in RalGDS translocation to the plasma membrane, we found that AT_1R-induced membrane blebbing does not require RalGDS, RalA, or RalB protein expression. The activation of the AT_1R 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 AT_1R-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 AT_1R 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 AT_1R-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 AT_1R-stimulated membrane blebbing. In contrast, RalGDS depletion by siRNA has no effect on AT_1R-stimulated membrane blebbing. β-Arrestin1 has been shown previously to activate RhoA-dependent stress fiber formation in response to AT_1R, 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 AT_1R 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

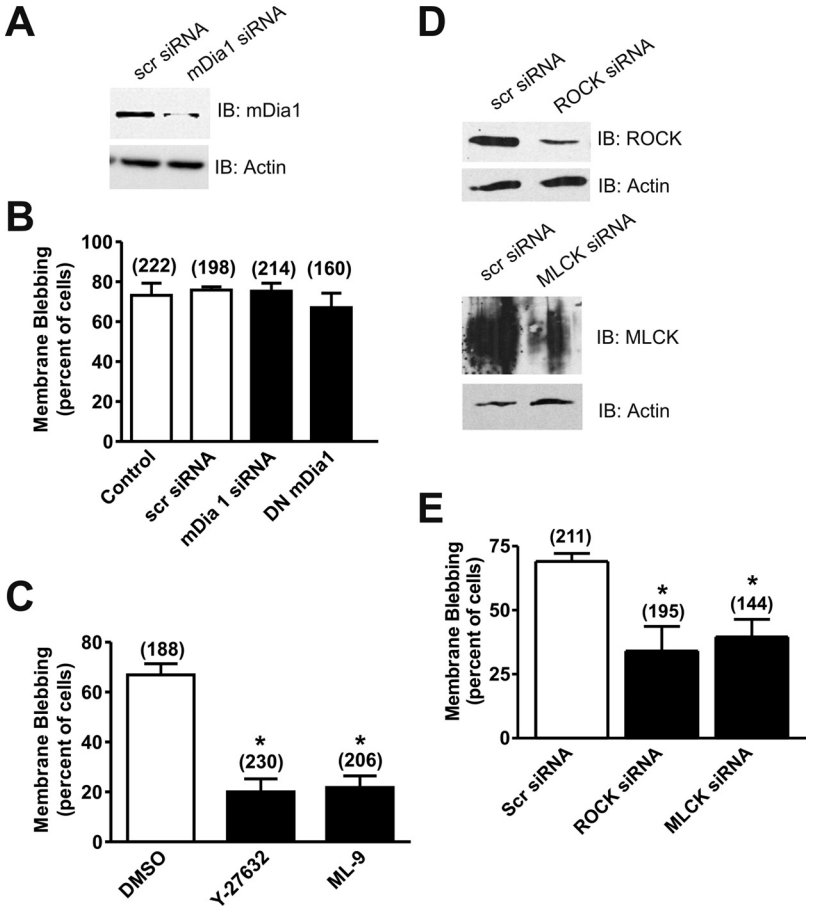

Fig. 7. Inhibition of MLCK and ROCK blocks AT_1R-induced membrane blebbing. A, shown is a representative autoradiograph of siRNA-mediated depletion of endogenous mDia1 protein expression in cells transfected with 5 μg of pcDNA3.1 cDNA plasmid encoding FLAG-AT_1R and treated with 100 pmol of either scrambled or mDia1 siRNAs. B, graph showing the percentage of HEK293 cells transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R that exhibit membrane blebbing responses in response treatment with 100 nM AngII when cotransfected with 100 pmol of either scrambled or mDia1-specific siRNA or transfected with 5 μg of pcDNA3.1 encoding a dominant-negative mDia1 (amino acid residues 736-1181Δ750-770) mutant. Data represent the mean ± S.E.M. of four independent experiments, and the numbers above the columns indicate the number of cells scored. C, graph showing the percentage of HEK293 cells transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R that exhibit membrane blebbing responses in response treatment with 100 nM AngII after a 1-h pretreatment with either DMSO, ROCK inhibitor (Y-27632, 10 μM), or MLCK inhibitor (ML-9, 50 μM). Data represent the mean ± S.E.M. of four 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 HEK293 cells expressing 5 μg of pcDNA3.1 cDNA plasmid encoding FLAG-AT_1R and treated with 100 pmol of either scrambled (scr), ROCK, or MLCK siRNAs. E, the percentage of HEK293 cells transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R that exhibit membrane blebbing responses in response to treatment with 100 nM AngII when cotransfected with 100 pmol of either scrambled, ROCK or MLCK siRNAs. Data represent the mean ± S.E.M. of four independent experiments, and the numbers above the columns indicate the number of cells scored. Asterisks indicate significant differences compared with control ($p < 0.05$). IB, immunoblot.

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 AT_1R results in $G\alpha_{q/11}$-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 AT_1R stimulation (Policha et al., 2006). However, the treatment of HEK293 cells with a variety of PKC inhibitors failed to block AT_1R-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 (Meshki et al., 2009). Likewise, PKA activity did not contribute to membrane blebbing. However, although Bis I, chelerythrine, Gö6976, and Gö6983 failed to block AT_1R-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 AT_1R-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 AT_1R-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 AT_1R. This is consistent with previous studies with the neurokinin 1 receptor and the observation that constitutively active ROCK induces bleb formation in Walter carcinoma cells, which is inhibited by the MLCK inhibitor ML-7 (Gutjahr et al., 2005; Meshki 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 AT_1R 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 AT_1R 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 AT_1R leads to plasma membrane blebbing responses by activation of RhoA and subsequent coupling to the ROCK/MLCK pathway.

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References

Albright CF, Giddings BW, Liu J, Vito M, and Weinberg RA (1993) Characterization of a guanine nucleotide dissociation stimulator for a ras-related GTPase. *EMBO J* **12:**339–347.

Aziziyeh AI, Li TT, Pape C, Pampillo M, Chidiac P, Possmayer F, Babwah AV, and Bhattacharya M (2009) Dual regulation of lysophosphatidic acid (LPA1) receptor signalling by Ral and GRK. *Cell Signal* **21:**1207–1217.

Barnes WG, Reiter E, Violin JD, Ren XR, Milligan G, and Lefkowitz RJ (2005) beta-Arrestin 1 and Galphaq/11 coordinately activate RhoA and stress fiber formation following receptor stimulation. *J Biol Chem* **280:**8041–8050.

Bhattacharya M, Anborgh PH, Babwah AV, Dale LB, Dobransky T, Benovic JL, Feldman RD, Verdi JM, Rylett RJ, and Ferguson SS (2002) Beta-arrestins regulate a Ral-GDS Ral effector pathway that mediates cytoskeletal reorganization. *Nat Cell Biol* **4:**547–555.

Calò LA and Pessina AC (2007) RhoA/Rho-kinase pathway: much more than just a modulation of vascular tone. Evidence from studies in humans. *J Hypertens* **25:**259–264.

Charras G and Paluch E (2008) Blebs lead the way: how to migrate without lamellipodia. *Nat Rev Mol Cell Biol* **9:**730–736.

Charras GT (2008) A short history of blebbing. *J Microsc* **231:**466–478.

Charras GT, Hu CK, Coughlin M, and Mitchison TJ (2006) Reassembly of contractile actin cortex in cell blebs. *J Cell Biol* **175:**477–490.

Charras GT, Yarrow JC, Horton MA, Mahadevan L, and Mitchison TJ (2005) Non-equilibration of hydrostatic pressure in blebbing cells. *Nature* **435:**365–369.

Chhabra ES and Higgs HN (2007) The many faces of actin: matching assembly factors with cellular structures. *Nat Cell Biol* **9:**1110–1121.

Cotton M, Boulay PL, Houndolo T, Vitale N, Pitcher JA, and Claing A (2007) Endogenous ARF6 interacts with Rac1 upon angiotensin II stimulation to regulate membrane ruffling and cell migration. *Mol Biol Cell* **18:**501–511.

Feig LA (2003) Ral-GTPases: approaching their 15 minutes of fame. *Trends Cell Biol* **13:**419–425.

Gutjahr MC, Rossy J, and Niggli V (2005) Role of Rho, Rac, and Rho-kinase in phosphorylation of myosin light chain, development of polarity, and spontaneous migration of Walker 256 carcinosarcoma cells. *Exp Cell Res* **308:**422–438.

Hofer F, Fields S, Schneider C, and Martin GS (1994) Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. *Proc Natl Acad Sci USA* **91:**11089–11093.

Hunton DL, Barnes WG, Kim J, Ren XR, Violin JD, Reiter E, Milligan G, Patel DD,

and Lefkowitz RJ (2005) Beta-arrestin 2-dependent angiotensin II type 1A receptor-mediated pathway of chemotaxis. *Mol Pharmacol* **67:**1229–1236.
Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri P, Davis MI, Edeen PT, et al. (2008) A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol* **26:**127–132.
Kikuchi A and Williams LT (1996) Regulation of interaction of ras p21 with RalGDS and Raf-1 by cyclic AMP-dependent protein kinase. *J Biol Chem* **271:**588–594.
Krebs A, Rothkegel M, Klar M, and Jockusch BM (2001) Characterization of functional domains of mDia1, a link between the small GTPase Rho and the actin cytoskeleton. *J Cell Sci* **114:**3663–3672.
Kurokawa K, Nakamura T, Aoki K, and Matsuda M (2005) Mechanism and role of localized activation of Rho-family GTPases in growth factor-stimulated fibroblasts and neuronal cells. *Biochem Soc Trans* **33:**631–634.
Lammers M, Meyer S, Kühlmann D, and Wittinghofer A (2008) Specificity of interactions between mDia isoforms and Rho proteins. *J Biol Chem* **283:**35236–35246.
Matrougui K, Tankó LB, Loufrani L, Gorny D, Levy BI, Tedgui A, and Henrion D (2001) Involvement of Rho-kinase and the actin filament network in angiotensin II-induced contraction and extracellular signal-regulated kinase activity in intact rat mesenteric resistance arteries. *Arterioscler Thromb Vasc Biol* **21:**1288–1293.
Matsubara K, Kishida S, Matsuura Y, Kitayama H, Noda M, and Kikuchi A (1999) Plasma membrane recruitment of RalGDS is critical for Ras-dependent Ral activation. *Oncogene* **18:**1303–1312.
Meshki J, Douglas SD, Lai JP, Schwartz L, Kilpatrick LE, and Tuluc F (2009) Neurokinin 1 receptor mediates membrane blebbing in HEK293 cells through a Rho/Rho-associated coiled-coil kinase-dependent mechanism. *J Biol Chem* **284:**9280–9289.
Policha A, Daneshtalab N, Chen L, Dale LB, Altier C, Khosravani H, Thomas WG, Zamponi GW, and Ferguson SS (2006) Role of angiotensin II type 1A receptor phosphorylation, phospholipase D, and extracellular calcium in isoform-specific protein kinase C membrane translocation responses. *J Biol Chem* **281:**26340–26349.
Shi Y, Zhang J, Mullin M, Dong B, Alberts AS, and Siminovitch KA (2009) The mDial formin is required for neutrophil polarization, migration, and activation of the LARG/RhoA/ROCK signaling axis during chemotaxis. *J Immunol* **182:**3837–3845.
Takai Y, Sasaki T, and Matozaki T (2001) Small GTP-binding proteins. *Physiol Rev* **81:**153–208.
Touyz RM (2005) Intracellular mechanisms involved in vascular remodelling of resistance arteries in hypertension: role of angiotensin II. *Exp Physiol* **90:**449–455.
Touyz RM and Schiffrin EL (2000) Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev* **52:**639–672.
Vemuri GS, Zhang J, Huang R, Keen JH, and Rittenhouse SE (1996) Thrombin stimulates wortmannin-inhibitable phosphoinositide 3-kinase and membrane blebbing in CHRF-288 cells. *Biochem J* **314:**805–810.
Weir MR and Dzau VJ (1999) The renin-angiotensin-aldosterone system: a specific target for hypertension management. *Am J Hypertens* **12:**205S–213S.
Wolthuis RM and Bos JL (1999) Ras caught in another affair: the exchange factors for Ral. *Curr Opin Genet Dev* **9:**112–117.
Yanase Y, Carvou N, Frohman MA, and Cockcroft S (2010) Reversible bleb formation in mast cells stimulated with antigen is Ca^{2+}/calmodulin-dependent and bleb size is regulated by ARF6. *Biochem J* **425:**179–193.

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