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The Small GTPase Ral Couples the Angiotensin II Type 1 Receptor to the Activation of Phospholipase C-δ1

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ABSTRACT

The angiotensin II type 1 receptor (AT_1R) plays an important role in cardiovascular function and as such represents a primary target for therapeutic intervention. The AT_1R has traditionally been considered to be coupled to the activation of phospholipase C (PLC) β via its association with $G\alpha_{q/11}$, leading to increases in intracellular inositol phosphate (IP) and release of calcium from intracellular stores. In the present study, we investigated whether the small GTPase RalA contributed to the regulation of AT_1R endocytosis and signaling. We find that neither RalA nor RalB is required for the endocytosis of the AT_1R, but that RalA expression is required for AT_1R-stimulated IP formation but not 5-HT_{2A} receptor-mediated IP formation. AT_1R-activated IP formation is lost in the absence of Ral guanine nucleotide dissociation stimulator (RalGDS), and requires the β-arrestin-dependent plasma membrane translocation of RalGDS. $G\alpha_{q/11}$ small interfering RNA (siRNA) treatment also significantly attenuates both AT_1R- and 5-HT_{2A} receptor-stimulated IP formation after 30 min of agonist stimulation. PLC-δ1 has been reported to be activated by RalA, and we show that AT_1R-stimulated IP formation is attenuated after PLC-δ1 siRNA treatment. Taken together, our results provide evidence for a G protein-coupled recepto-activated and RalGDS/Ral-mediated mechanism for PLC-δ1 stimulation.

The angiotensin II type 1 receptor (AT_1R) is a member of the G protein-coupled receptor (GPCR) superfamily and regulates cellular function in a variety of tissues (de Gasparo et al., 2000). In the vasculature, angiotensin II (AngII)-mediated stimulation of the AT_1R activates a myriad of cell signaling cascades that regulate vascular smooth muscle cell tone, growth, apoptosis, migration, and extracellular matrix deposition (Ushio-Fukai et al., 1998; Touyz and Schiffrin, 2000; Haendeler et al., 2003). The AT_1R activates the $G\alpha_{q/11}$ class of heterotrimeric G proteins that stimulate phospholipase C, leading to diacylglycerol and inositol-1,4,5-trisphosphate (IP_3) formation, the release of Ca^{2+} from intracellular stores, and subsequent activation of protein kinase C. There are six families of phospholipase C isozymes (PLC-β, PLC-γ, PLC-δ, PLC-ε, PLC-ζ, and PLC-η) that generate diacylglycerol and IP_3 from phosphatidylinositol 4, 5-bisphosphate (PIP_2) (Suh et al., 2008). The AT_1R has traditionally been demonstrated to activate PLC-β but can also induce tyrosine phosphorylation of PLC-γ1 leading to the production of IP_3 (Lea et al., 2002; Ochocka and Pawelczyk, 2003; Suh et al., 2008).

Although the membrane receptors involved in the activation of PLC-δ have not been clearly delineated, there is some evidence that GPCRs may be coupled to PLC-δ activation. The activation of α1-adrenergic and oxytocin receptors has been reported to lead to the activation of PLC-δ via an interaction with $G\alpha_h$ (transglutaminase II) (Feng et al., 1996, 1999; Park et al., 1998). In addition, bradykinin receptor-mediated capacitive calcium entry after the activation of PLC-β has been reported to increase PLC-δ responses in PC12 cells overexpressing the phospholipase (Kim et al.,

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ABBREVIATIONS: AT_1R, angiotensin II type 1 receptor; GPCR, G protein-coupled receptor; AngII, angiotensin II; IP_3, inositol-1,4,5-trisphosphate; PLC, phospholipase C; CaM, calmodulin; mGluR, metabotropic glutamate receptor; IP, inositol phosphate; siRNA, small interfering RNA; RalGDS, Ral guanine nucleotide dissociation stimulator; HEK, human embryonic kidney; BRET, bioluminescence resonance energy transfer; IP, inositol phosphate; GFP, green fluorescent protein; YFP, yellow fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; 5-HT, serotinin; 5-HT_{2A}R, serotinin 2A receptor; DMSO, dimethyl sulfoxide.

1999) However, the mechanism(s) underlying the activation of PLC-δ and the coupling of this enzyme to membrane receptors remains poorly understood.

In a recent study, a novel PLC-δ regulatory mechanism involving the direct interaction of the small GTPases RalA and RalB and calmodulin (CaM) was reported (Sidhu et al., 2005). Ral proteins were reported to bind to and activate PLC-δ, whereas CaM binding to a novel IQ motif identified within the catalytic domain of PLC-δ inhibited PLC-δ activity. Moreover, it was demonstrated that Ral binding to PLC-δ alone was sufficient to activate the phospholipase in vitro and that this was independent of guanine nucleotide state of the GTPase.

Ral GTPases are now recognized to be involved in various aspects of GPCR function, such as the regulation of signaling, endocytosis, and changes in cytoskeletal organization (Bhattacharya et al., 2004a). A role for GPCRs in the activation of RalA was first described for the fMet-Leu-Phe receptor (M'Rabet et al., 1999) Subsequently, it has been shown that fMet-Leu-Phe receptor mediated activation of Ral requires the β-arrestin-dependent plasma membrane translocation of green fluorescent protein (GFP)-RalGDS, which is required for Ral activation (Bhattacharya et al., 2002). Ral also interacts with Group I metabotropic glutamate receptors (mGluRs) to regulate the phospholipase D2-dependent endocytosis of both mGluR1 and mGluR5 (Bhattacharya et al., 2004b). Ral has also recently been implicated in the regulation of lysophosphatidic acid receptor 1-stimulated inositol phosphate (IP) formation (Aziziyeh et al., 2009). In the present study, we examined whether RalA and RalB might contribute to the regulation of AT_1R endocytosis and signaling. We report that AT_1R-stimulated IP formation is regulated by a RalGDS- and RalA-specific mechanism that involves the activation of PLC-δ. Taken together, our data describe a new mechanism by which GPCRs can be coupled to the activation of PLC-δ.

Materials and Methods

Materials. [*myo*-^{3}H]Inositol was acquired from PerkinElmer Life and Analytical Sciences (Waltham, MA). The Dowex 1-X8 (formate form) resin with 200 to 400 mesh was purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). Bovine serum albumin was obtained from BioShop Canada Inc. (Mississauga, ON, Canada). siRNAs were purchased from Dharmacon RNA Technologies (Lafayette, CO). RalB, RalGDS, $G\alpha_{q/11}$, PLC-δ and actin antibodies were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). RalA antibody was purchased from BD Transduction Laboratories (Mississauga, ON, Canada). Horseradish peroxidase-conjugated anti-rabbit and anti-goat IgG secondary antibodies were from Bio-Rad Laboratories. β-Arrestin antibody was a gift from Dr. Stephane Laporte (Royal Victoria Hospital, Montreal, QC, Canada). ECL Western blotting detection reagents were from Thermo Fisher Scientific (Waltham, MA). Horseradish peroxidase-conjugated anti-mouse IgG secondary antibody was purchased from GE Healthcare (Oakville, ON, Canada). Lipofectamine 2000 and Alexa Fluor 555 antibody labeling reagents were purchased from Invitrogen (Burlington, ON, Canada). Rabbit anti-FLAG antibody and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Transfection. HEK293 cells were cultured in minimal essential medium 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 transfected using a modified calcium phosphate method (Ferguson and Caron, 2004), with the amount of cDNA indicated. Media was replaced 16 h after transfection. For use of siRNA, cells were seeded into 60-mm dishes and transfected using Lipofectamine 2000 from Invitrogen.

siRNA Transfection. We used siRNA against human RalA GGACUACGCUGCAAUUAGAUU modified from Lalli and Hall (2005), RalB CGCUUCAGUUCAUGUAUGAUU, RalGDS CCAUCUUCCUGUGUACCUA, PLC-δ1 AAGGUGGAAGUCCAGCUCAUGGA modified from Stallings et al. (2008), and $G\alpha_{q/11}$ AAGAUGUUCGUGGACCUGAACUU (Barnes et al., 2005). 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 analysis. An HEK293 cell line carrying TET-inducible shRNA targeting both β-arrestin isoforms was transfected using a modified calcium phosphate method and treated with 1 mg/ml doxycycline as described previously (Zimmerman et al., 2009).

Confocal Microscopy. Confocal microscopy was performed using a Zeiss LSM-510 META laser-scanning microscope equipped with a Zeiss 63× oil immersion lens. Live cell imaging was performed on cells in 35-mm glass-bottomed plates. Cells were kept in Hank's balanced salt solution at 37°C using a heated microscope stage. AT_1R was labeled with rabbit anti-FLAG conjugated Zenon Alexa Fluor 555 antibody. Receptor was stimulated with addition of 100 nM (final concentration) of AngII. Visualization of Alexa Fluor 555 antibody labeled AT_1R with either GFP-RalA or yellow fluorescent protein (YFP)-Ral and β-Arrestin1-GFP was performed by dual excitation (488, 543 nm) and resolved by spectral analysis using the emission fingerprinting function of the LSM-510 META (Carl Zeiss Inc., Thornwood, NY). Semiquantitative analysis of GFP-RalGDS translocation was determined as follows; multiple images were obtained over time, AngII was added during imaging to a final concentration of 100 nM. Cells were scored for translocation when a reduction in fluorescence intensity was recorded in the cytoplasm and results were expressed as a percentage of total cells scored expressing GFP-RalGDS.

Bioluminescent Resonance Energy Transfer. Transfected HEK293 cells were washed with PBS and incubated in trypsin for 1 min then quenched in 5 ml of minimal essential medium containing 8% fetal bovine serum. Cells were removed from the dish and placed in centrifuge tubes and centrifuged at 1800*g* for 5 min to pellet the cells. The cells were washed by resuspending in PBS and recentrifuged. Cells were resuspended in 1 ml of BRET buffer (1% glucose and 1 μM ascorbic acid in PBS) containing the protease inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, and leupeptin. Protein concentration was measured by spectrophotometry using a detergent-compatible protein assay (Bio-Rad Laboratories). Cell suspensions were diluted in BRET buffer to make a final protein concentration of 1 μg/μl. Cells were placed in a 96-well plate, 50 μl per well, and 25 μl of 15 μM coelenterazine H was added followed by agonist stimulation with 100 nM final concentration of AngII or serotonin. Fluorescence was read on a Victor3 (PerkinElmer Life and Analytical Sciences) using 460 and 535 nm filters. The BRET ratio was calculated as described previously (Holmes et al., 2006).

Internalization Assay. Cells expressing the indicated cDNA were split in quadruplicate in 24-well plates. Cells were incubated in binding mixture comprising either serum-free DMEM containing 10 mM HEPES, 50 μCi/ml ^{125}I-angiotensin II (total binding), or in the same binding mixture also containing 50 μM losartan (nonspecific binding) on ice for 3 h. Plates were washed three times in serum-free DMEM supplemented with 10 mM HEPES. Cells were placed at 37°C and receptor was allowed to internalize for 30 min. These plates were then cooled on ice to stop internalization and incubated in acid wash (50 mM sodium citrate and 90 mM NaCl, pH 5.0) for 20 min to remove bound radioligand from the cell surface. Acid wash was removed by three rinses in serum free DMEM + 10 mM HEPES. Cells were solubilized in 0.1 N NaOH and transferred to test tubes. The control plate was not warmed to 37°C and did not receive acid wash; this provided the total of cell surface ^{125}I-angiotensin II binding sites. Radioactivity was counted on a gamma counter (Beckman Coulter, Fullerton, CA). Specific binding and percentage internalization was calculated.

Inositol Phosphate Formation Assay. Agonist-dependent inositol phosphate formation was determined as described previously

(Dale et al., 2001). In brief, cells were incubated overnight in 1 μCi/ml [*myo*-^{3}H]inositol in DMEM to radiolabel cellular inositols. Excess [*myo*-^{3}H]inositol was removed by washing in HBSS, followed by a 1-h incubation in HBSS and a subsequent 10-min incubation in HBSS containing 10 mM LiCl. Cells were then stimulated for 30 min with either AngII or 5-HT at concentrations indicated in the figures. The reaction was stopped on ice with the addition of 0.8 M perchloric acid followed by neutralization with 0.72 M KOH/0.6 M $KHCO_3$. Total [^{3}H]inositol incorporated into the cell was determined by counting the radioactivity of 50 μl of cell lysate. Inositol phosphates were purified from the cell lysates using AG 1-X8 (formate form) anion exchange resin 200 to 400 mesh (Bio-Rad Laboratories). [^{3}H]Inositol phosphate formation was determined by liquid scintillation using a scintillation system (LS 6500; Beckman Coulter). Cells treated with calmidazolium chloride were incubated in 30 μM inhibitor for 10 min with 10 mM LiCl; this was replaced with the same mixture for an additional 30 min.

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), goat anti-RalGDS (1:500), rabbit anti-$G\alpha_{q/11}$ (1:1000), rabbit anti-PLC-δ (1:1000), or rabbit anti-actin (1:1000) 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 ECL Western blotting detection reagents.

Data Analysis. Statistical significance was determined either by analysis of variance testing followed by post hoc multiple comparison testing or by paired *t* test.

Results

AT_1R Colocalization and Association with RalA. We previously demonstrated that RalA interacted with group I mGluRs and facilitated the internalization of these receptors through the activation of PLD2 (Ferguson and Caron, 2004). Internalization of the AT_1R was also previously reported to be mediated by a PLD2-dependent mechanism (Albright et al., 1993). Therefore, we examined whether a FLAG-AT_1R construct might colocalize with and associate with RalA. When expressed in HEK293 cells, FLAG-AT_1R was expressed at the cell surface of transfected cells (Fig. 1A). GFP-tagged RalA expressed in the same cell was also membrane localized and to a lesser extent within intracellular vesicular structures (Fig. 1A). In response to agonist stimulation (100 nM AngII), Alexa Fluor 555-labeled FLAG-AT_1R was internalized into large homotypically fused endosomal structures, where it was extensively colocalized with both β-arrestin2-GFP and YFP-RalA (Fig. 1B).

To further explore a potential interaction between the AT_1R and RalA, we performed BRET analysis using pRluc with either YFP-RalA or β-arrestin1-YFP (negative controls), FLAG-AT_1R-Rluc and β-arrestin1-YFP (positive control) and FLAG-AT_1R-Rluc and YFP-RalA. In cells expressing pRluc with either YFP-RalA or β-arrestin1-YFP, no BRET ratio was obtained, and this ratio was not altered by agonist treatment (Fig. 2). In contrast, in cells expressing FLAG-AT_1R-Rluc and β-arrestin1-YFP, agonist stimulation with 100 nM AngII increased the BRET ratio from 0.09 $\pm$ 0.03 to 0.29 $\pm$ 0.01 (Fig. 2). A BRET ratio of 0.33 $\pm$ 0.05 was observed in cells expressing FLAG-AT_1R-Rluc and YFP-RalA in the absence of agonist stimulation, whereas agonist treatment reduced the BRET ratio slightly to 0.26 $\pm$ 0.02 (Fig. 2). The significant BRET ratio between FLAG-AT_1R-Rluc and YFP-RalA was consistent with the confocal data showing that RalA and AT_1R were colocalized at the plasma membrane and suggested a constitutive interaction between the AT_1R and RalA. Consequently, we examined whether Ral GTPases contributed to the regulation of AT_1R by specifically knocking down the expression of either RalA or RalB using siRNA. Treatment of cells with RalA siRNA specifically knocked down the expression of RalA without affecting the expression of RalB but did not alter the extent of FLAG-AT_1R internalization after exposure to AngII (100 nM) for 30 min (Fig. 3A).

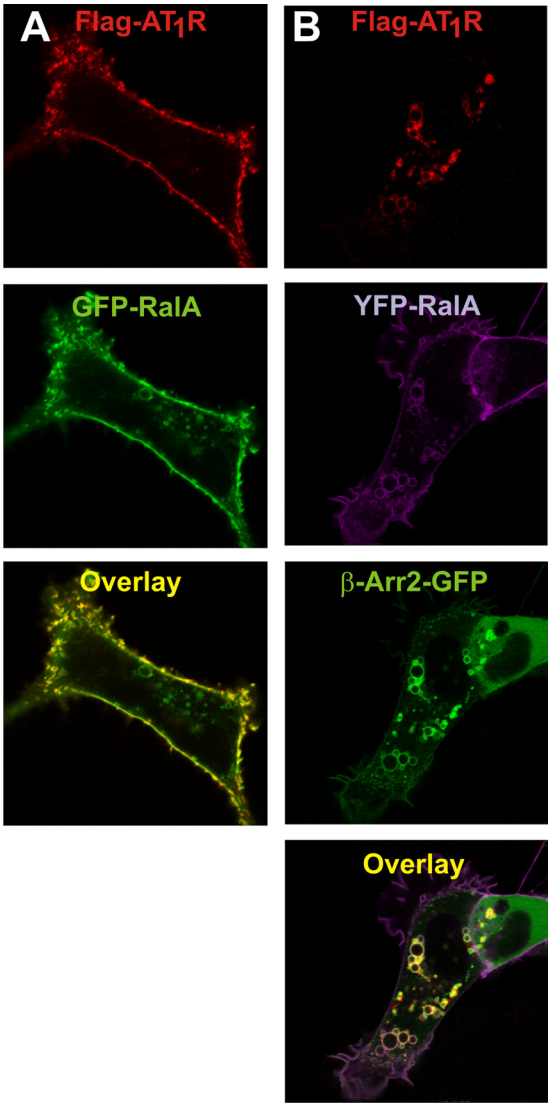

Fig. 1. AT_1R colocalizes with RalA. A, representative confocal micrographs of HEK293 cells transiently transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and 2 μg of peGFP-C1 plasmid cDNA encoding GFP-RalA, and labeled with 555 rabbit-anti FLAG antibody conjugated to Alexa Fluor 555 to stain cell-surface FLAG-AT_1R in the absence of agonist treatment. The confocal images presented are representative images from three independent experiments. B, representative confocal micrographs of HEK293 cells transiently transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R, 2.5 μg of peGFP-N1 plasmid cDNA encoding GFP-β-arrestin 2, and 1 μg of peYFPC1 plasmid cDNA encoding YFP-RalA. Cells were labeled with 555 rabbit-anti FLAG antibody conjugated to Alexa Fluor 555 to stain cell surface FLAG-AT_1R and then treated with 100 nM AngII for 30 min. The confocal images presented are representative images from three independent experiments.

Likewise, treatment of cells with RalB-specific siRNA knocked down the expression of RalB without affecting the expression of RalA, but also did not alter the extent of FLAG-AT_1R internalization (Fig. 3B). Thus, RalA and RalB do not seem to contribute to the endocytosis of the AT_1R.

RalA-Dependent AT_1R-Stimulated IP Formation. Because we found that neither RalA nor RalB contributed to the regulation of AT_1R internalization, we examined whether RalA and RalB were involved in AT_1R-mediated IP formation. To test this, we measured agonist-stimulated IP production in HEK293 cells expressing FLAG-AT_1R and treated with scrambled siRNA (control) or siRNAs directed against RalA and RalB (Fig. 4A). In FLAG-AT_1R-expressing cells treated with RalA siRNA, the maximum response to increasing concentrations of AngII was reduced to 53 ± 6.2% of control (Fig. 4B). In contrast, FLAG-AT_1R-mediated IP formation in response to increasing AngII concentrations was unaffected by RalB knockdown (Fig. 4C). To examine the specificity of RalA-dependent IP formation, we examined whether RalA siRNA treatment would also alter serotonin 2A receptor (5-$HT_{2A}R$)-stimulated IP formation. When tested, we found that RalA expression was not required for 5-$HT_{2A}R$-stimulated IP formation (Fig. 4D). Thus, RalA seemed to selectively contribute to the regulation of AT_1R-stimulated IP formation.

RalGDS-Dependent Activation of PLC. We have demonstrated previously that the activation of Ral in response to fMLPR activation involved the β-arrestin-dependent translocation of RalGDS to the plasma membrane (Bhattacharya et al., 2002). Therefore, we examined whether RalGDS translocation and/or expression was required for AT_1R stimulated IP formation. Initial experiments examined whether the expression of the β-arrestin amino terminus would prevent the plasma membrane translocation of RalGDS in a manner similar to what we previously reported for the fMLPR (Bhattacharya et al., 2002). In cells cotransfected with FLAG-AT_1R and GFP-RalGDS, GFP-RalGDS was localized to the cytoplasm and, in response to AngII (100 nM) treatment, redistributed to the plasma membrane in the same cells in 73 ± 6.2% of cells (Fig. 5, A and B). However, coexpression of the β-arrestin amino terminus reduced the number of cells exhibiting GFP-RalGDS translocation responses to 46 ± 16% (Fig. 5B). To test whether RalGDS was involved in AT_1R-stimulated IP formation, HEK293 cells were treated with siRNA to reduce RalGDS protein expression (Fig. 5C). The treatment of cells with RalGDS siRNA reduced IP formation stimulated by AngII (100 nM) to 39 ± 9.5% of control (Fig. 5D). Likewise, the overexpression of the β-arrestin amino terminus to prevent RalGDS translocation reduced IP formation stimulated by AngII (100 nM) to 58 ± 7.7% of control (Fig. 5E). To further test the involvement of β-arrestins, we used an HEK293 cell line stably expressing a TET-inducible shRNA targeting both β-arrestins 1 and 2. Cells were transfected with FLAG-AT_1R and treated with or without doxycycline to induce knock-down of β-arrestin expression (Fig. 5F). This resulted in an increase in IP production in treated cells (Fig. 5G). Thus, the data indicate that RalGDS expression and translocation was required for AT_1R-stimulated IP formation.

AT_1R-Stimulated IP Formation Is $G\alpha_{q/11}$ Dependent. To investigate the mechanism by which RalGDS and Ral

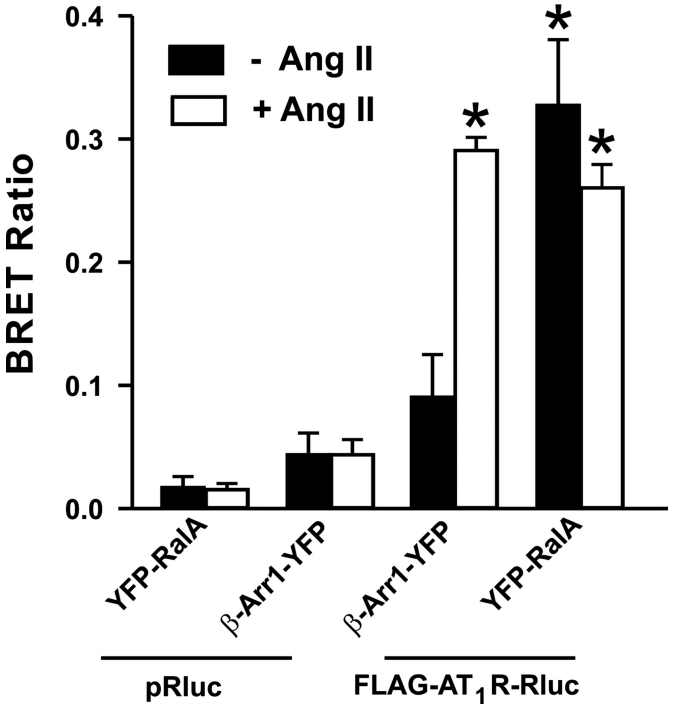

Fig. 2. Assessment of FLAG-AT_1R-Rluc interactions with YFP-RalA by BRET. BRET ratios were determined for protein-protein interactions in HEK293 cells transfected with 2 μg of empty pRluc plasmid cDNA along with 2 μg of either peYFP-C1plasmid cDNA encoding YFP-RalA or peYFP-N1 plasmid cDNA encoding β-arrestin1-YFP (negative controls), 0.25 μg of pRluc plasmid cDNA encoding FLAG-AT_1R-pRluc along with 2 μg of peYFP-N1 plasmid cDNA encoding β-arrestin1-YFP (positive control), and 0.25 μg of pRluc plasmid cDNA encoding FLAG-AT_1R-pRluc along with 0.5 μg of peYFP-C1 plasmid cDNA encoding YFP-RalA in either the absence or presence of AngII (100 nM) for 5 min. *, $p < 0.05$. Data are mean BRET ratio ± S.E.M. of four independent experiments.

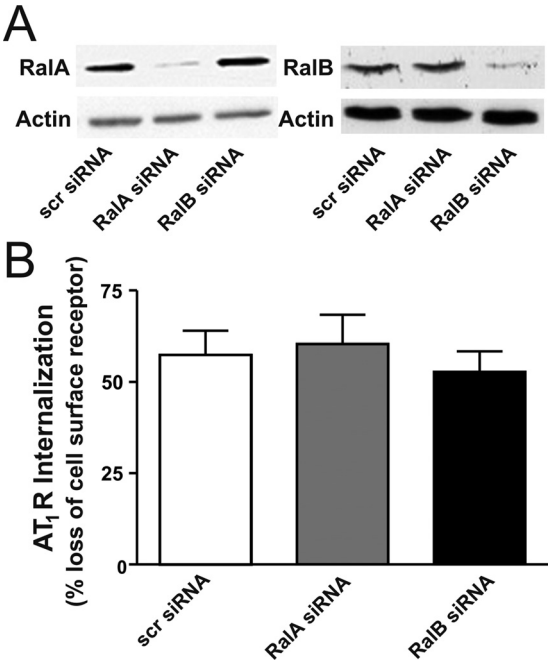

Fig. 3. Effect of RalA and RalB siRNA treatment on FLAG-AT_1R internalization. A, representative immunoblots for endogenous RalA and RalB protein as well as actin expression in HEK293 cells transfected with 1 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and treated with 100 pmol of either scrambled, RalA-specific, or RalB-specific siRNA. B, shown is the loss of cell surface FLAG-AT_1R expression in HEK293 cells transfected with 1 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and treated with 100 pmol of either scrambled, RalA-specific, or RalB-specific siRNA after the treatment of HEK293 cells with 100 nM AngII for 30 min at 37°C. Data represent the mean ± S.E.M. of eight independent experiments.

contributed to the regulation of AT_1R-stimulated IP formation, we first tested whether siRNA-mediated reduction of $G\alpha_{q/11}$ protein expression would reduce AT_1R-stimulated IP formation as expected. The knockdown of $G\alpha_{q/11}$ using an siRNA that was previously shown to attenuate $G\alpha_{q/11}$-mediated Rho activation (Barnes et al., 2005) attenuated AT_1R-stimulated IP formation in HEK293 cells to 28 ± 13% of control when treated with AngII for 30 min (Fig. 6A). Likewise, when cells were treated with $G\alpha_{q/11}$ siRNA, IP formation in response to the activation of another $G\alpha_{q/11}$-coupled GPCR, the 5-HT_{2A}R, was significantly reduced to 52 ± 12.8% of control (Fig. 6B). Consequently, the activation of IP formation in response to AT_1R activation was $G\alpha_{q/11}$-mediated.

AT_1R-Stimulated IP Formation Is PLC-δ1-Dependent. The observation that siRNA-mediated knockdown of RalA significantly attenuates AT_1R-mediated IP formation suggested that RalA coupled the receptor to an alternative PLC isoform. Sidhu et al. (2005) reported that RalA interacted directly with PLC-δ1 and was sufficient to activate the phospholipase in vitro. Moreover, this effect of RalA was independent of the guanine nucleotide state of the GTPase. Therefore, we tested whether siRNA knockdown of PLC-δ1 expression would affect AT_1R-stimulated IP formation. Consistent with a role of PLC-δ1 in mediating AT_1R signaling, siRNA knockdown of PLC-δ1 significantly attenuated agonist-stimulated (100 nM) AT_1R IP formation from 44.5 ± 8.4% to 14 ± 2.7 and to 39.8 ± 11.7% of control at 15 and 30 min, respectively. There was no significant difference after 5-min treatment with 100 nM AngII (Fig. 7A). The activity of PLC-δ1 has previously been demonstrated to be inhibited by the association of CaM with a novel IQ motif identified within the catalytic domain of PLC-δ1 in a Ral-dependent manner. Therefore, we examined the effect of treating HEK293 cells with CaM inhibitor calmidazolium chloride on basal and agonist-stimulated IP formation in the absence and presence of transfected FLAG-AT_1R. The treatment of empty plasmid transfected cells for 10 min with 30 μM calmidazolium chloride resulted in a significant increase in [*myo*-^{3}H]inositol conversion to [^{3}H]IP over vehicle (DMSO)-treated control cells (Fig. 7B). In cells transfected to express FLAG-AT_1R, basal IP formation induced by calmidazolium chloride treatment was further increased to 6.9 ± 1.7% from 2.1 ± 0.6% in the absence of receptor expression (Fig. 7B). In response to agonist treatment, FLAG-AT_1R-dependent IP formation was increased to a similar extent in vehicle versus calmidazolium chloride-treated cells, indicating that the drug treatment does not effect the maximum extent of IP formation in response to agonist. To confirm that the observed increase in basal IP formation in the presence of the CaM inhibitor was dependent on PLC-δ1 activity, we examined basal IP formation in cells expressing FLAG-AT_1R and treated with PLC-δ1 siRNA. In cells treated with siRNA basal [*myo*-^{3}H]inositol conversion to [^{3}H]IP was not statistically significantly increased over DMSO-treated control cells after calmidazolium chloride treatment (Fig. 7B). Thus, the data indicate that CaM played a role in AT_1R-stimulated activation of PLC-δ1.

Discussion

In the present study, we investigated the role of Ral GTPases in the regulation of AT_1R endocytosis and signaling. Although we found that neither RalA nor RalB contributes to the regulation of AT_1R endocytosis, we did find that RalA and not RalB is specifically involved in the coupling of the AT_1R to the activation of IP_3 signal transduction. This RalA-medi-

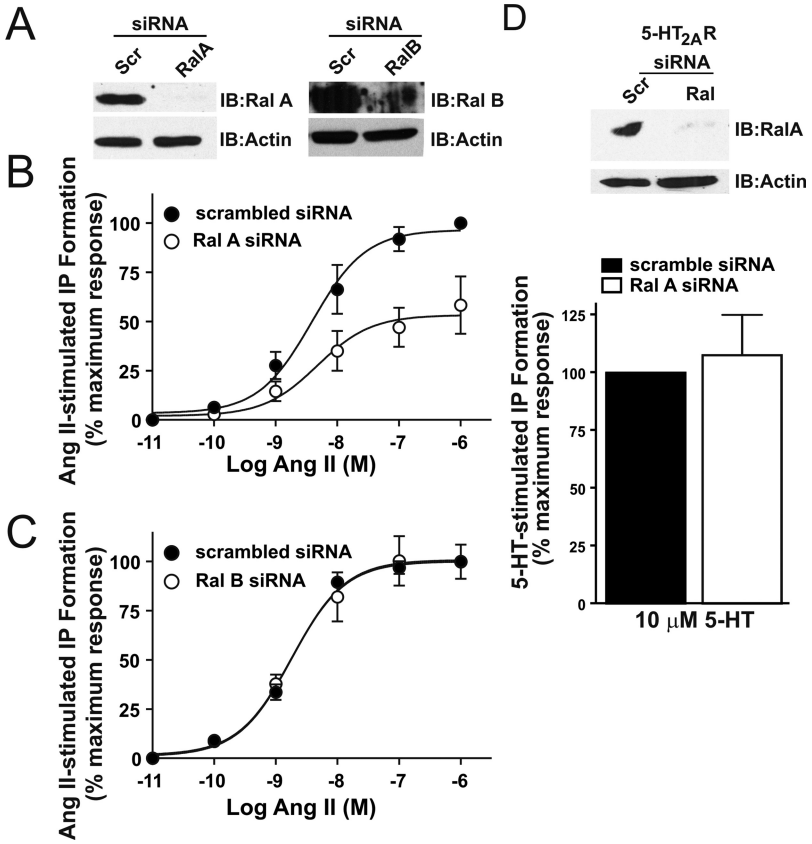

Fig. 4. Effect of Ral siRNA treatment on FLAG-AT_1R- and 5-HT_{2A}R-stimulated IP formation. A, representative immunoblots for endogenous RalA and RalB as well as actin protein expression in HEK293 cells transfected with 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and treated with 100 pmol of either scrambled, RalA-specific, or RalB-specific siRNA. B, FLAG-AT_1R-stimulated IP formation in response to increasing concentrations of AngII (10^{-11}–10^{-6} M) for 30 min at 37°C in HEK293 cells transfected with 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and treated with 100 pmol of either scrambled or RalA-specific siRNA. Data represent the mean ± S.E.M. of five independent experiments. C, FLAG-AT_1R-stimulated IP formation in response to increasing concentrations of AngII (10^{-11}–10^{-6} M) for 30 min at 37°C in HEK293 cells transfected with 1 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and treated with 100 pmol of either scrambled or RalB-specific siRNA. Data represent the mean ± S.E.M. of four independent experiments. D, top, immunoblots for endogenous $G\alpha_{q/11}$ and actin protein expression in HEK293 cells transfected with 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-5-HT_{2A}R and treated with 100 pmol of either scrambled or RalA-specific siRNA. Graph shows the effect of RalA-specific siRNA treatment on FLAG-5-HT_{2A}R-stimulated IP formation in response to treatment with 10 μM 5-HT for 30 min at 37°C. The data represent the mean ± S.E.M. of five independent experiments. *, $p < 0.05$.

ated activation of IP_3-mediated signaling seems to require the activation of RalGDS protein translocation to the plasma membrane and is also dependent upon RalGDS protein expression. Moreover, although IP formation in response to AT_1R is dependent upon the activation of the $G\alpha_{q/11}$ signaling pathway, AT_1R-stimulated IP formation is also mediated by PLC-δ1. In contrast, IP formation in response to the activation of the 5-$HT_{2A}R$ receptor is dependent upon $G\alpha_{q/11}$ protein expression but does not involve RalA. We also propose that PLC-δ1 activity is likely to involve concomitant roles for both RalA and CaM. Taken together, our data provide a new mechanism linking the activation of GPCRs to the stimulation of PLC-δ1-mediated IP_3 formation.

RalA and RalB share 85% amino acid content (Oxford et al., 2005) and yet can have divergent cellular functions. For example, RalA and RalB have nonoverlapping and opposing functions in cancer cell migration but overlapping functions in cell growth (Oxford et al., 2005). RalGDS binds to both RalA and RalB and facilitates the exchange of GDP for GTP on these proteins (Albright et al., 1993). This specificity for RalA over RalB is consistent with their distinct roles in Ras-mediated malignant transformations (Oxford et al., 2005; Lim et al., 2006). In the present study, we show that depletion of RalA protein from HEK293 cells significantly reduces IP production in response to AT_1R activation. However, depletion of RalB has no effect on AT_1R signaling. We found that depletion of RalGDS also inhibits IP production of agonist-stimulated AT_1R. This suggests that RalA activity is necessary for activation of PLC-δ1. This differs from the in vitro work of Sidhu et al. (2005), who demonstrated that, similar to what is observed for Ral interactions with PLD, RalA interacts with PLC-δ1 irrespective of its GDP/GTP state (Luo et al., 1997, 1998). Thus, we suggest that AT_1R activation results in the β-arrestin-dependent translocation of RalGDS to the plasma, where it associates with the

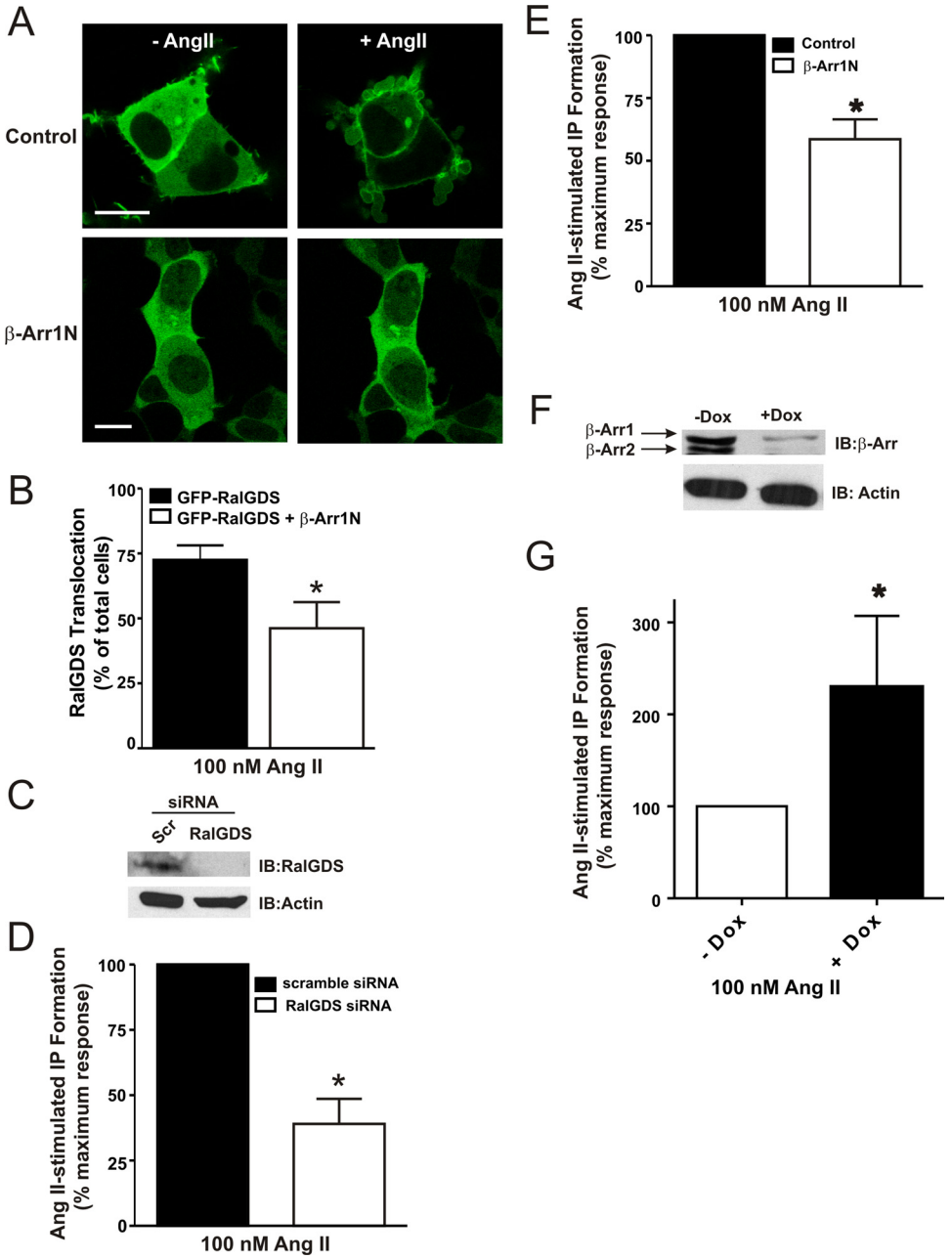

Fig. 5. Effect of β-ArrN1 on GFP-RalGDS translocation and RalGDS siRNA and β-ArrN1 FLAG-AT_1R-stimulated IP formation. A, representative confocal micrographs of HEK293 cells transiently transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and 1 μg of peGFP-C2 plasmid cDNA encoding GFP-RalGDS along with either 5 μg of empty pRK5 plasmid cDNA or 5 μg of pRK5 plasmid cDNA encoding the β-arrestin1 N terminus (β-ArrN1). Micrographs show the subcellular localization of GFP-RalGDS protein either before (−AngII) or after treatment of the same cells with 100 nM AngII for 5 min (−AngII). B, data represent percentage of HEK293 cells exhibiting GFP-RalGDS translocation in response to 100 nM over a 10-min time course in 71 independent live cells imaged in the absence of β-Arr1N and 83 independent live cells imaged in the presence of β-ArrN1 in five independent experiments. C, a representative immunoblot for endogenous RalGDS and actin protein expression in HEK293 cells transfected with 1 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and treated with 100 pmol of RalGDS-specific siRNA. D, FLAG-AT_1R-stimulated IP formation in response to treatment with 100 nM AngII for 30 min at 37°C in HEK293 cells transfected with 1 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and treated with 100 pmol of RalGDS-specific siRNA. Data represent the mean ± S.E.M. of four independent experiments. E, FLAG-AT_1R-stimulated IP formation in response to treatment with 100 nM AngII for 30 min at 37°C in HEK293 cells transfected with 1 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and 5 μg of pRK5 cDNA encoding β-ArrN1. F, a representative immunoblot for endogenous β-arrestin 1 and 2 and actin protein in HEK293 cells transfected with 3 mg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and treated with 1 mg/ml doxycycline. G, FLAG-AT_1R-stimulated IP formation in response to treatment with 100 nM Angil for 30 min at 37°C in HEK293 cells transfected with 3 mg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R. Data represent the mean ± S.E.M. of four independent experiments. *, $p < 0.05$.

AT_1R/RalA complex and promotes the RalA-dependent activation of PLC-δ1.

The RalA-dependent activation of PLC-δ1 is also calcium-dependent and seems to involve the calcium-dependent release of CaM from PLC-δ1 (Sidhu et al., 2005). Previous studies have implicated PLC-δ1 as an effector for oxytocin, α_{1B}-adrenergic, and bradykinin receptors (Park et al., 1998; Feng et al., 1999; Kim et al., 1999). In the case of the bradykinin receptor, the activation of PLC-δ1 is dependent upon the influx of extracellular calcium (Kim et al., 1999), whereas the activation of PLC-δ1 by the oxytocin and α_{1B}-adrenergic receptors involves the formation of a complex containing $G\alpha_h$ (Park et al., 1998; Feng et al., 1999; Baek et al., 2001). In the present study, we find that the treatment of the AT_1R for 30 min with AngII resulted in IP formation that is blocked by both $G\alpha_{q/11}$ and PLC-δ1 siRNA treatment. The AT_1R stimulates PLC-β-mediated IP formation and the release of Ca^{2+} from intracellular stores as well as the influx of extracellular

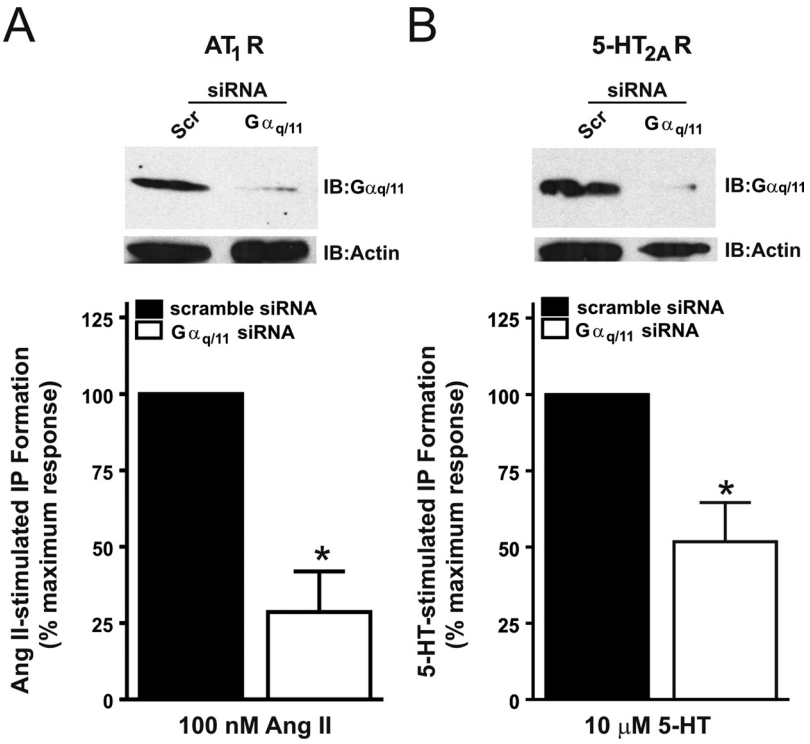

Fig. 6. Role of $G\alpha_{q/11}$ in FLAG-AT_1R- and FLAG-5-$HT_{2A}R$-stimulated IP formation. A, top, immunoblots for endogenous $G\alpha_{q/11}$ and actin protein expression in HEK293 cells transfected with 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and treated with 100 pmol of either scrambled or $G\alpha_{q/11}$-specific siRNA. Graph shows the effect of $G\alpha_{q/11}$-specific siRNA treatment on FLAG-AT_1R-stimulated IP formation in response to treatment with 100 nM AngII for 30 min at 37°C. The data represent the mean ± S.E.M. of four independent experiments. B, top, immunoblots for endogenous $G\alpha_{q/11}$ and actin protein expression in HEK293 cells transfected with 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-5-$HT_{2A}R$ and treated with 100 pmol of either scrambled or $G\alpha_{q/11}$-specific siRNA. Graph shows the effect of $G\alpha_{q/11}$-specific siRNA treatment on FLAG-5-$HT_{2A}R$-stimulated IP formation in response to treatment with 10 μM 5-HT for 30 min at 37°C. The data represent the mean ± S.E.M. of four independent experiments.

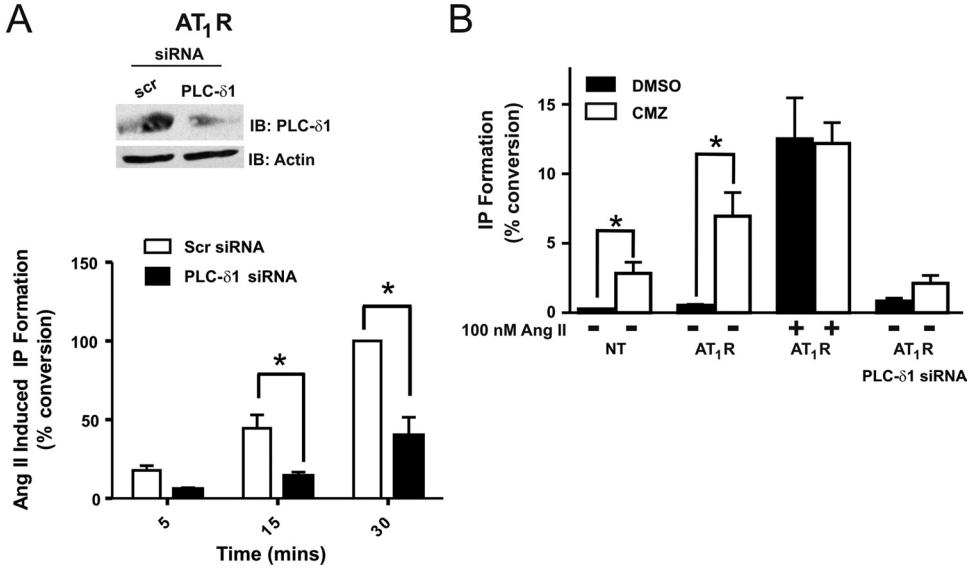

Fig. 7. Effect of PLC-δ1 siRNA and calmidazolium chloride treatment on FLAG-AT_1R-stimulated IP formation. A, top, immunoblots for endogenous PLC-δ1 protein and actin expression in HEK293 cells transfected with 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R and treated with 100 pmol of either scrambled or PLC-δ1-specific siRNA. Graph shows the effect of PLC-δ1-specific siRNA treatment on FLAG-AT_1R-stimulated IP formation in response to treatment with 100 nM AngII for 30 min at 37°C. The data represent the mean ± S.E.M. of five independent experiments. B, effect of calmidazolium chloride (CMZ) treatment (30 μM in 0.3% by volume DMSO) in HEK293 cells transfected with either 2 μg of empty pcDNA3.1 plasmid cDNA, 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R, or 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT_1R treated with 100 pmol of PLC-δ1-specific siRNA. Cells were treated with CMZ for 10 min before measuring [^{3}H]IP formation for 20 min in either the absence or presence of agonist. Cells were treated either with or without agonist as indicated. Graphs show mean ± S.E.M. for four independent experiments. *, $p < 0.05$. NT, nontransfected cells.

calcium (Ushio-Fukai et al., 1998; Touyz and Schiffrin, 2000; de Gasparo et al., 2000; Haendeler et al., 2003; Policha et al., 2006). We propose that the initial coupling of the AT_1R to PLC-β stimulates IP_3-dependent increases in intracellular Ca^{2+} concentrations that might in part also contribute to the subsequent Ca^{2+} -dependent activation of PLC-δ1 signaling by the AT_1R. However, the coupling of the AT_1R to PLC-β would be expected to become uncoupled in response to β-arrestin binding and the dissociation of the receptor from $G\alpha_{q/11}$. RalGDS translocation to the plasma membrane would then lead to the RalA-dependent release of the PLC-δ1 CaM block, resulting in the activation of IP formation via PLC-δ1.

The activation of PLC-δ1 by the AT_1R may be important for normal physiological cardiac function, because PLC-δ1 activity and expression is dysregulated in rat models of cardiac disease (Tappia et al., 2001; Asemu et al., 2003; Hwang et al., 2004). Specifically, PLC-δ1 protein expression is decreased in ischemic heart and hypoxic neonatal cardiomyocytes Hwang et al., 2004; Asemu et al., 2003). After ischemia, PLC-δ, but not PLC-β or PLC-γ, is degraded by calcium-sensitive proteases (Hwang et al., 2004). This has led to the suggestion that changes in PLC isozyme expression may contribute to alterations in calcium homeostasis in myocardial ischemia. Moreover, PLC-δ1 activation is an important target for tumor necrosis factor receptor-mediated protection against cardiac injury (Lien et al., 2006). Consistent with a potential role for PLC-δ1 in cardiac protection administration of the angiotensin-converting enzyme inhibitor imidapril partially increases sarcolemmal PLC activity (Tappia et al., 1999). Thus, alterations in the coupling of AT_1R to PLC-δ1 may be relevant to cardiovascular disease.

In summary, we report that RalA is localized with and is trafficked with the AT_1R but does not contribute to the regulation of AT_1R endocytosis. AT_1R activation leads to the plasma membrane translocation of RalGDS, which is required for the Ral-dependent activation of PLC-δ1. Taken together, our data identify the AT_1R as a membrane receptor for PLC-δ1 and RalA as the transducer for PLC-δ1.

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