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THE SMALL GTPASE RAL COUPLES THE ANGIOTENSIN II TYPE 1 RECEPTOR TO THE ACTIVATION OF PHOSPHOLIPASEC-δ1

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Running title: RalA couples the AT_1R to the activation of PLCδ1

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Abbreviations: ANOVA, analysis of variance; AngII, angiotensin II; AT_1R, angiotensin type 1 receptor; CaM, calmodulin; GFP, green fluorescence protein; GPCR, G protein-coupled receptor; HEK 293 cells, human embryonic kidney cells, IP, inositol phosphate; PLC, phospholipase C; siRNA, RalGDS, Ral guanine nucleotide dissociation stimulator, small interfering RNA.

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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 Cβ 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 are 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. $G\alpha_{q/11}$ siRNA treatment also significantly attenuates both AT_1R-5-HT_{2A} receptor stimulated IP formation following 30 minutes of agonist stimulation. PLC-δ1 has been reported to be activated by RalA and we show here that AT_1R-stimulated IP formation is attenuated following PLC-δ1 siRNA treatment. Taken together, our results provide evidence for a GPCR activated and RalGDS/Ral-mediated mechanism for PLC-δ1 stimulation.

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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; Haendeler, et al., 2003; Touyz and Schiffrin, 2000). The AT_1R activates the $G\alpha_{q/11}$ class of heterotrimeric G proteins that stimulate phospholipase C leading to diacylglycerol (DAG) 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 DAG and IP_3 from phosphatidylinositol 4, 5-bisphosphate (PIP_2) (Shu, et al., 2008). Classically, the AT_1R has been demonstrated to activate PLC-β, but can also induce tyrosine phosphorylation of PLC-γ1 leading to the production of IP_3 (Shu, et al., 2008; Ochocka and Pawelczyk, 2003; Lea, et al., 2002).

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., 1999; Park, et al., 1998). In addition, bradykinin receptor-mediated capacitive calcium entry following the activation of PLC-β has been reported to increase PLC-δ responses in PC12 cells overexpressing the phospholipase (Kim, et al., 1999) However,

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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 G protein couple receptor (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 (FMLP) receptor (M'Rabet, et al., 1999) Subsequently, it has been shown that fMLP receptor mediated activation of Ral requires the β-arrestin-dependent plasma membrane translocation of 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

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PLC-δ. Taken together, our data describe a new mechanism by which GPCRs can be coupled to the activation of PLC-δ.

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MATERIALS AND METHODS

Materials: *myo*-[^{3}H]Inositol was acquired from PerkinElmer Life Sciences (Waltham, MA). The Dowex 1-X8 (formate form) resin with 200–400 mesh was purchased from Bio-Rad (Mississauga, ON). Bovine serum albumin (BSA) was obtained from BioShop Canada Inc. (Mississauga, ON). siRNAs were purchased from Dharmacon RNAi Technologies. 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 (Mississauga, ON). Horseradish peroxidase-conjugated anti-rabbit and anti-goat IgG secondary antibodies were from BioRad (Mississauga, ON). ECL Western blotting detection reagents were from Thermo Scientific (Rockford, IL). Horseradish peroxidase-conjugated anti-mouse IgG secondary antibody was purchased from GE Healthcare (Oakville, ON). Lipofectamine 2000 and AlexaFluor 555 antibody labeling reagents were purchased from Invitrogen/Molecular Probes (Burlington, ON). Rabbit anti-FLAG antibody and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and Transfection: HEK 293 were cultured in minimal essential media (MEM) supplemented with 8% FBS (vol\vol) and 100μg/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 hours after transfection. For use of siRNA, cells were seeded into 60 mm dishes and transfected using Lipofectamine 2000 from Invitrogen.

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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 (Non-Targeting siRNA #1) was purchased from Dharmacon. Experiments were performed 48 or 72 hours after transfection and knock down of proteins was confirmed by western blot.

Confocal Microscopy: Confocal microscopy was performed using a Zeiss LSM-510 META laser scanning microscope equipped with a Zeiss 63x oil immersion lens. Live cell imaging was performed on cells in 35 mm glass bottom 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 Alexaflour 555 antibody. Receptor was stimulated with addition of 100 nM (final concentration) of Ang II. Visualization of AlexaFluor 555 antibody labeled AT_1R with either GFP-RalA or 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 Zeiss LSM-510 META. Semi-quantitative analysis of GFP-RalGDS translocation was determined as follows; multiple images were obtained over time, Ang II was added during imaging to a final concentration of 100 nM. Cells were scored for translocation when a reduction in

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fluorescence intensity was recorded in the cytoplasm and results were expressed as a percent of total cells scored expressing GFP-RalGDS.

Bioluminescent Resonance Energy Transfer: Transfected HEK 239 cells were washed with PBS and incubated in trypsin for 1 minute then quenched in 5 mL of MEM containing 8% FBS. Cells were removed from the dish and placed in centrifuge tubes and centrifuged at 1800 g for 5 minutes to pellet the cells. The cells were washed by resuspending in PBS and recentrifuged. Cells were resuspended in 1 mL BRET buffer (1% glucose and 1 μM ascorbic acid in PBS) containing the protease inhibitors, AEBSF, aprotinin and leupeptin. Protein concentration was measured by spectrophotometry using Dc protein assay (BioRad). 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 Ang II or serotonin. Fluorescence was read on a Victor3 (PerkinElmer), using 460 nm 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 either comprised of 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 (non-specific binding) on ice for 3 hours. Plates were washed 3 times in serum free DMEM supplemented with 10 mM HEPES. Cells were placed at 37°C and receptor was allowed to internalize for 30

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minutes. These plates were then cooled on ice to stop internalization and incubated in acid wash (50 mM sodium citrate, 90 mM NaCl, pH 5.0) for 20 minutes to remove bound radioligand from the cell surface. Acid wash was removed by 3 rinses in serum free DMEM + 10 mM HEPES. Cells were solubilized in 0.1N NaOH and transfered 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 Beckman γ counter. Specific binding and percent internalization was calculated.

Inositol Phosphate (IP) Formation Assay: Agonist dependent inositol phosphate formation was determined as previously described previously (Dale, et al., 2001). Briefly, cells were incubated over night 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 hour incubation in HBSS, and subsequently a 10 minute incubation in HBSS containing 10 mM LiCl . Cells were then stimulated with either Ang II or 5-HT at concentrations indicated in the Figures for 30 minutes. 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 200-400 mesh (Bio-Rad). [^{3}H]-inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system. Cells treated with calmidazolium chloride, were

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incubated in 30 μM inhibitor for 10 minutes with 10 mM LiCl, then this was replaced with the same mixture for an addition 30 minutes.

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 1h 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 (1:10000), donkey anti-mouse IgG (1:2500) and Rabbit anti-goat IgG (1:5000) in wash buffer containing 3% skim milk for 1h. 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 (ANOVA) testing followed by post-hoc Multiple Comparison testing or by paired T-Test.

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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). Green fluorescence protein (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 (AngII, 100 nM) AlexFluor 555-labelled FLAG-AT_1R was internalized into large homotypically fused endosomal structures where it was extensively colocalized with both β-arrestin2-GFP and yellow fluorescence protein (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 ± 0.03 to 0.29 ± 0.01 (Fig. 2). A BRET ratio of 0.33 ± 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 ± 0.02 (Fig. 2). The significant BRET ratio between FLAG-AT_1R-

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Rluc and YFP-RalA was consistent with the confocal data that showed 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 following exposure to AngII (100 nM) for 30 min (Fig. 3A). Similarly, 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 appear to contribute to the endocytosis of the AT_1R.

***RalA-dependent AT_1R-stimulated IP formation* -** Since we did not find that either RalA or 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 alter 5-$HT_{2A}R$-stimulated IP formation was also dependent upon RalA, we examined the effect

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of RalA siRNA treatment on 5-HT_{2A}R signaling. When tested, we found RalA expression was not required for 5-HT_{2A}R-stimulated IP formation (Fig. 4D). Thus, RalA appeared to selectively contribute to the regulation of AT_1R-stimulated IP formation.

***RalGDS-dependent activation of PLC* -** Previously, we demonstrated 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. 5A and 5B). 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 AngII (100 nM)-stimulated IP formation to 39 ± 9.5% of control (Fig. 5D). Similarly, the overexpression of the β-arrestin amino terminus to prevent RalGDS translocation reduced AngII (100 nM)-stimulated IP formation to 58 ± 7.7% of control (Fig. 5E). Thus, the data indicated that RalGDS expression and translocation was required for AT_1R-stimulated IP formation.

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AT_1R-stimulated IP formation is $G\alpha_{q/11}$ dependent - To investigate the mechanism by which RalGDS and Ral 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 $\pm$ 13% of control when treated with AngII for 30 min (Fig. 6A). Similariliy, 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 serotonin 2A receptor (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 attenuate AT_1R-mediated IP formation suggested that RalA coupled the receptor to an alternative PLC isoform then coupling of PLCβ through $G\alpha_{q/11}$. Previously, Sidhu *et. al.* (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 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 to 28 ± 7.2% of control (Fig. 7A). Previously, the activity of PLC-δ1 was demonstrated to be inhibited by the association of CaM with a novel IQ motif

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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 $[^3H]$myo-inositol conversion to $[^3H]$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 $[^3H]$myo-inositol conversion to $[^3H]$IP was not statistically significantly increased over vehicle (DMSO) treated control cells following calmidazolium chloride treatment (Fig. 7B). Thus, the data indicated that CaM played a role in AT_1R-stimulated activation of PLC-δ1.

Discussion

In the present manuscript we investigated the role of Ral GTPases in the regulation of AT_1R endocytosis and signaling. Although we did not find that either RalA or RalB contribute to the regulation of AT_1R endocytosis, we did find that RalA and not RalB is

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specifically involved in the coupling of the AT_1R to the activation of IP_3 signal transduction. This RalA mediated activation of IP_3-mediated signaling appears 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 likely involves 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.* (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

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GDP/GTP state (Luo, et al., 1998; Luo, et al., 1997). Thus, we suggest that AT_1R activation results in the β-arrestin-dependent translocation of RalGDS to the plasma where it associates with the 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 appears 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 (Feng, et al., 1999; Park, et al., 1998; 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 $G\alpha_h$ (Feng, et al., 1999; Park, et al., 1998; 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 calcium (de Gasparo, et al., 2000; Ushio-Fukai, et al., 1998; Haendeler, et al., 2003; Touyz and Schiffrin, 2000; 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

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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, as PLC-δ1 activity and expression is dysregulated in rat models of cardiac disease (Hwang, et al., 2004; Asemu, et al., 2003; Tappia, et al., 2001). Specifically, PLC-δ1 protein expression is decreased in ischemic heart and hypoxic neonatal cardiomyocytes Hwang, et al., 2004; Asemu, et al., 2003). Following 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 tumour 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 ACE 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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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: **AT_1R colocalizes with RalA.** **(A)** Shown are 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 AlexaFlour 555 to stain cell surface FLAG-AT_1R in the absence of agonist treatment. The confocal images presented are representative images from 3 independent experiments. **(B)** Shown are 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 AlexaFlour 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 3 independent experiments.

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Figure 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

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or presence of AngII (100 nM) for 5 min. * $p<0.05$. Data is mean BRET Ratio ± SEM of 4 independent experiments.

Figure 3: **Effect of RalA and RalB siRNA treatment on FLAG-AT$_1$R internalization.** **(A)** Shown are 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$_1$R and treated with 100 pmoles of either scrambled, RalA-specific or RalB-specific siRNA. **(B)** Shown is the loss of cell surface FLAG-AT$_1$R expression in HEK293 cells transfected with 1 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT$_1$R and treated with 100 pmoles of either scrambled, RalA-specific or RalB-specific siRNA following the treatment of HEK293 cells with 100 nM AngII for 30 min at 37°C. Data represents the mean ± SEM of 8 independent experiments.

Figure 4: **Effect of Ral siRNA treatment on FLAG-AT$_1$R-and 5-HT$_{2A}$R-stimulated IP formation.** **(A)** Shown are 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$_1$R and treated with 100 pmoles of either scrambled, RalA-specific or RalB-specific siRNA. **(B)** Shown is FLAG-AT$_1$R-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$_1$R and treated with 100 pmoles of either scrambled or RalA-specific siRNA. Data represents the mean +/- SEM of 5 independent experiments. **(C)** Shown is FLAG-AT$_1$R-stimulated IP formation in response to increasing concentrations of AngII

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(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 pmoles of either scrambled or RalB-specific siRNA. Data represents the mean ± SEM of 4 independent experiments. **(D)** Upper panel shows 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 pmoles 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 ± SEM of 5 independent experiments. * $p<0.05$.

Figure 5: **Effect of β-ArrN1 on GFP-RalGDS translocation and RalGDS siRNA and β-ArrN1 FLAG-AT_1R-stimulated IP formation. (A)** Shown are 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 prior to (- AngII) or following treatment of the same cells with 100 nM AngII for 5 min (+ AngII). **(B)** Data represents 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 5 independent experiments. **(C)** Shown is a representative immunoblot for endogenous RalGDS and actin protein expression in HEK293 cells transfected with 1 μg of pcDNA3.1 plasmid

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cDNA encoding FLAG-AT_1R and treated with 100 pmoles of RalGDS-specific siRNA. **(D)** Shown is 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 pmoles of RalGDS-specific siRNA. Data represents the mean +/- SEM of 4 independent experiments. **(E)** Shown is 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. Data represents the mean ± SEM of 4 independent experiments. * $p<0.05$.

Figure 6: **Role of $G\alpha_{q/11}$ in FLAG-AT_1R- and FLAG-5-$HT_{2A}R$-stimulated IP formation. (A)** Upper panel shows 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 pmoles 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 ±SEM of 4 independent experiments. **(B)** Upper panel shows 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 pmoles 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 ±SEM of 4 independent experiments.

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Figure 7: **Effect of PLC-δ1 siRNA and calmidazolium chloride treatment on FLAG-AT_1R-stimulated IP formation. (A)** Upper panel shows 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 pmoles 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 ± SEM of 5 independent experiments. **(B)** Shows 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 pmoles of PLC-δ1-specific siRNA. Cells were treated with CMZ for 10 min prior to 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 in the Figure Panel. Graphs show mean ± SEM for 4 independent experiments. * $p<0.05$, (NT), Non-transfected cells (NT).

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Figure 1

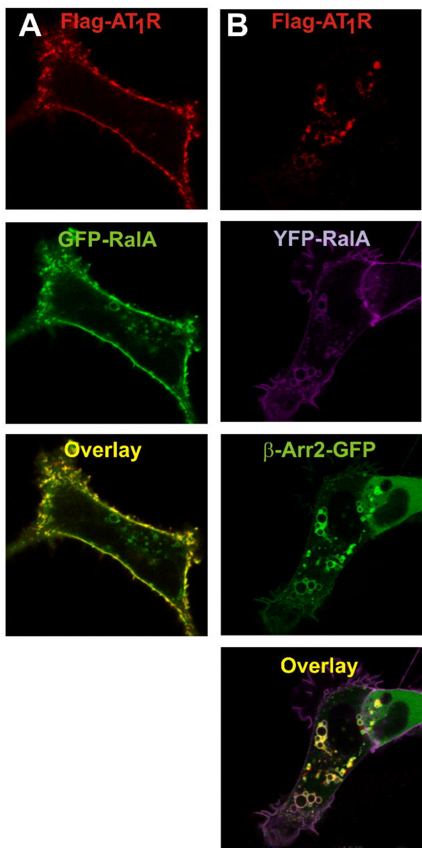


Figure 2

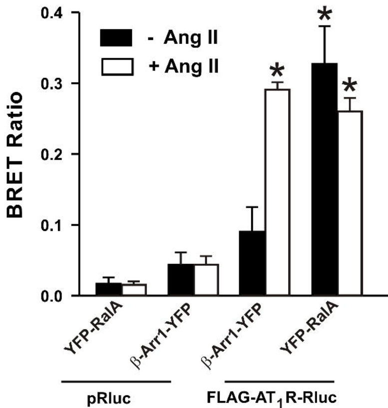


Figure 3

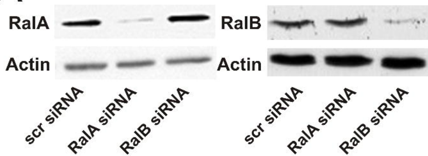


Figure 4

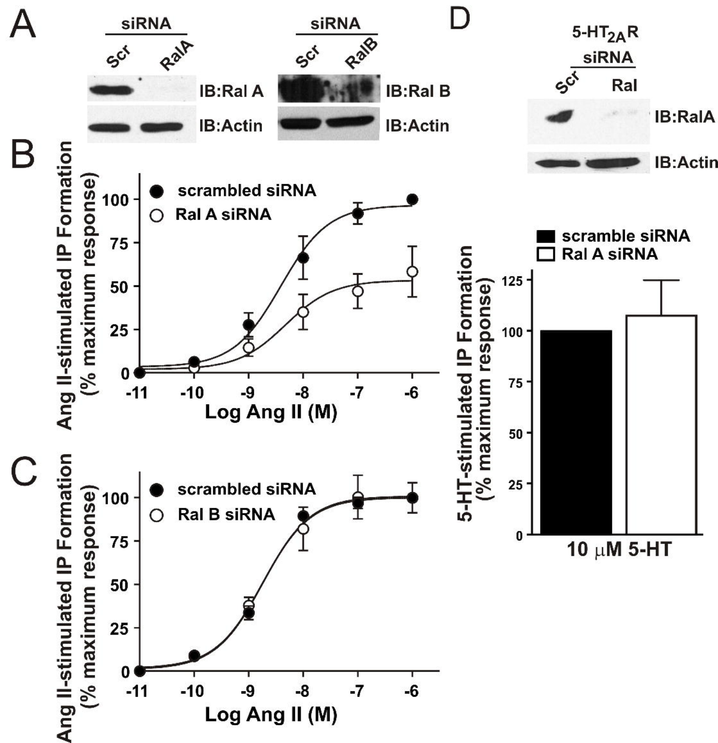
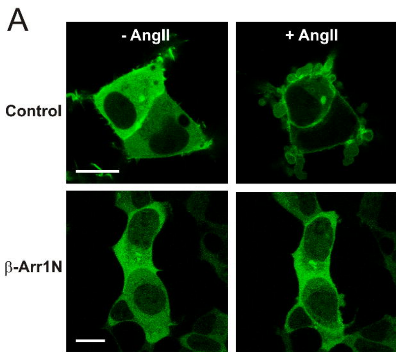
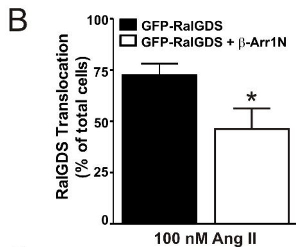
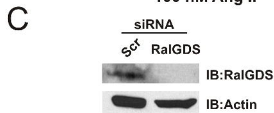
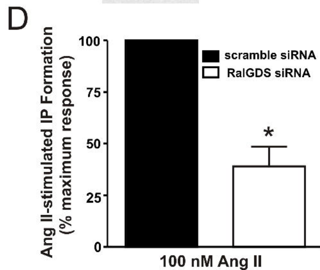


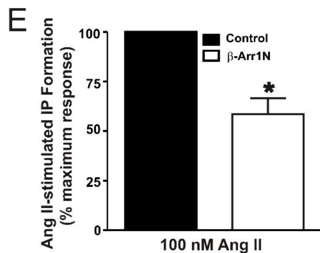
Figure 5

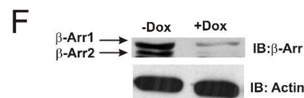












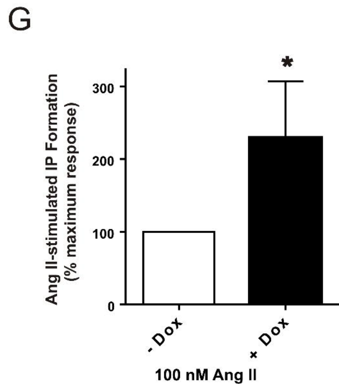
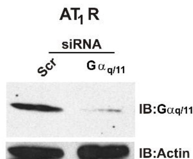
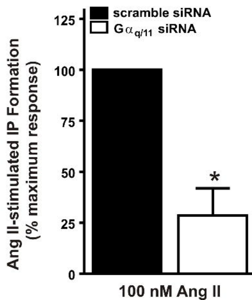


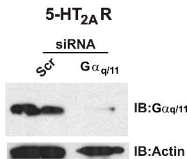
Figure 6

A





B



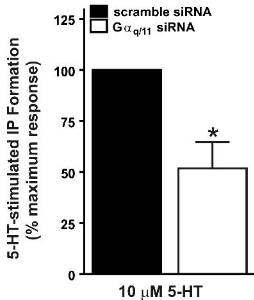


Figure 7

A

AT$_1$R

siRNA

scr PLC-δ1

IB: PLC-δ1

IB: Actin

Scr siRNA

PLC-δ1 siRNA

Ang II Induced IP Formation (% conversion)

150

100

50

0

5 15 30

Time (mins)