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 (AT1R) plays an important role in cardiovascular function and as such represents a primary target for therapeutic intervention. The AT1R has traditionally been considered to be coupled to the activation of phospholipase C (PLC) β via its association with Goq/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 AT1R endocytosis and signaling. We find that neither RalA nor RalB is required for the endocytosis of the AT1R, but that RalA expression is required for AT1R-stimulated IP formation but not 5-HT2A receptor-mediated IP formation.

AT1R-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. Goq/11 small interfering RNA (siRNA) treatment also significantly attenuates both AT1R- and 5-HT2A receptor-stimulated IP formation after 30 min of agonist stimulation. PLC-δ1 has been reported to be activated by RalA, and we show that AT1R-stimulated IP formation is attenuated after PLC-δ1 siRNA treatment. Taken together, our results provide evidence for a G protein-coupled receptor-activated and RalGDS/Ral-mediated mechanism for PLC-δ1 stimulation.
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 GTPasesRalA 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 metotropic 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 phospholipid (IP) formation (Azzizyeh et al., 2009). In the present study, we examined whether RalA and RalB might contribute to the regulation of AT,R endocytosis and signaling. We report that AT,R-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-3H]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, Goα11, PLC-δ and actin antibodies were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). RalA antibody was purchased from BD Transduction Laboratories (Burlington, ON, Canada). Horse serum, peroxidase-conjugated anti-rabbit and anti-goat IgG secondary antibodies were from Bio-Rad Laboratories. β-Arrestin antibody was a gift from Dr. Stephens Laporte (Royal Victoria Hospital, Montreal, QC, Canada). ECL Western blotting detection reagents were from Thermo Fisher Scientific (Waltham, MA). Horseradish peroxidase-conjugated antimouse 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 5% fetal bovine serum (v/v) and 100 μg/ml gentamicin. All reagents were obtained from Invitrogen. Cells were seeded into 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 GCCUGCUAGAAAGAUU modified from Stallings et al. (2008), and Goα11 AAGAUGUUGCGACCUGAACUU (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-induced shRNA expressing 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,R was labeled with rabbit anti-FLAG conjugated Zenon Alexa Fluor 555 antibody. RalB was labeled with antibody anti-FLAG conjugated Zenon Alexa Fluor 555 antibody labeled AT,R with either GFP-RalA or yellow fluorescent protein (YFP)-Ral and β-Arrestin-1-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 1800g 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/ml. Cells were placed in a 96-well plate, 5 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 [125I]-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 [125I]-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 KHCO3. 
Total [3H]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). [3H]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 
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-Gαq (1:1000), 
rabbit anti-PLC-β (1:1000), or rabbit anti-actin (1:1000) antibodies in wash buffer containing 5% 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 5% 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**

**AT1R 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 AT1R was also previously reported to be 
mediated by a PLD2-dependent mechanism (Albright et al., 1993). Therefore, we examined whether a FLAG-AT1R construct might colocalize with and associate with RalA. 
When expressed in HEK293 cells, FLAG-AT1R 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-AT1R 
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 
AT1R and RalA, we performed BRET analysis using pRluc 
with either YFP-RalA or β-arrestin1-YFP (negative controls), 
FLAG-AT1R-Rluc and β-arrestin1-YFP (positive control) and 
FLAG-AT1R-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-AT1R-
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-AT1R-Rluc and YFP-RalA in the 
absence of agonist stimulation, whereas agonist treatment re-
duced the BRET ratio slightly to 0.26 ± 0.02 (Fig. 2). The 
significant BRET ratio between FLAG-AT1R-Rluc and YFP-
RalA was consistent with the confocal data showing that 
RalA and AT1R were colocalized at the plasma membrane and 
suggested a constitutive interaction between the AT1R and 
RalA. Consequently, we examined whether Ral GTPases 
contributed to the regulation of AT1R 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-AT1R internalization 
after exposure to AngII (100 nM) for 30 min (Fig. 3A).
Likewise, treatment of cells with RaIB-specific siRNA knocked down the expression of RaIB without affecting the expression of RaIA, but also did not alter the extent of FLAG-AT1R internalization (Fig. 3B). Thus, RaIA and RaIB do not seem to contribute to the endocytosis of the AT1R.

**RalA-Dependent AT1R-Stimulated IP Formation.** Because we found that neither RaIA nor RaIB contributed to the regulation of AT1R internalization, we examined whether RaIA and RaIB were involved in AT1R-mediated IP formation. To test this, we measured agonist-stimulated IP production in HEK293 cells expressing FLAG-AT1R and treated with scrambled siRNA (control) or siRNAs directed against RaIA and RaIB (Fig. 4A). In FLAG-AT1R-expressing cells treated with RaIA siRNA, the maximum response to increasing concentrations of AngII was reduced to 53 ± 6.2% of control (Fig. 4B). In contrast, FLAG-AT1R-mediated IP formation in response to increasing AngII concentrations was unaffected by RaIB knockdown (Fig. 4C). To examine the specificity of RaA-dependent IP formation, we examined whether RaIA siRNA treatment would also alter serotonin 2A receptor (5-HT2AR)-stimulated IP formation. When tested, we found that RaIA expression was not required for 5-HT2AR-stimulated IP formation (Fig. 4D). Thus, RaIA seemed to selectively contribute to the regulation of AT1R-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 AT1R 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-AT1R 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 AT1R-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-AT1R 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 AT1R-stimulated IP formation.

**AT1R-Stimulated IP Formation Is Goα11 Dependent.** To investigate the mechanism by which RalGDS and Ral
contributed to the regulation of AT\textsubscript{1}R-stimulated IP formation, we first tested whether siRNA-mediated reduction of Go\textsubscript{q/11} protein expression would reduce AT\textsubscript{1}R-stimulated IP formation as expected. The knockdown of Go\textsubscript{q/11} using an siRNA that was previously shown to attenuate Go\textsubscript{q/11}-mediated Rho activation (Barnes et al., 2005) attenuated AT\textsubscript{1}R-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 Go\textsubscript{q/11} siRNA, IP formation in response to the activation of another Go\textsubscript{q/11}-coupled GPCR, the 5-HT\textsubscript{2A}R, was significantly reduced to 52 ± 12.8% of control (Fig. 6B). Consequently, the activation of IP formation in response to AT\textsubscript{1}R activation was Go\textsubscript{q/11}-mediated.

AT\textsubscript{1}R-Stimulated IP Formation Is PLC-61-Dependent. The observation that siRNA-mediated knockdown of RalA significantly attenuates AT\textsubscript{1}R-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-61 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-61 expression would affect AT\textsubscript{1}R-stimulated IP formation. Consistent with a role of PLC-61 in mediating AT\textsubscript{1}R signaling, siRNA knockdown of PLC-61 significantly attenuated agonist-stimulated (100 nM) AT\textsubscript{1}R 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-61 has previously been demonstrated to be inhibited by the association of CaM with a novel IQ motif identified within the catalytic domain of PLC-61 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\textsubscript{1}R. The treatment of empty plasmid transfected cells for 10 min with 30 μM calmidazolium chloride resulted in a significant increase in [myo-\textsuperscript{3}H]inositol conversion to [\textsuperscript{3}H]IP over vehicle (DMSO)-treated control cells (Fig. 7B). In cells transfected to express FLAG-AT\textsubscript{1}R, 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\textsubscript{1}R-dependent IP formation was increased to a similar extent in vehicle versus calmidazolium chloride-treated cells, indicating that the drug treatment does not affect 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-61 activity, we examined basal IP formation in cells expressing FLAG-AT\textsubscript{1}R and treated with PLC-61 siRNA. In cells treated with siRNA basal [\textsuperscript{3}H]inositol conversion to [\textsuperscript{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\textsubscript{1}R-stimulated activation of PLC-61.

**Discussion**

In the present study, we investigated the role of Ral GTPases in the regulation of AT\textsubscript{1}R endocytosis and signaling. Although we found that neither RalA nor RalB contributes to the regulation of AT\textsubscript{1}R endocytosis, we did find that RalA and not RalB is specifically involved in the coupling of the AT\textsubscript{1}R to the activation of IP\textsubscript{3} signal transduction. This RalA-medi-
ated activation of IP₃-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 AT₁R is dependent upon the activation of Gₛq₁₁ signaling pathway, AT₁R-stimulated IP formation is also mediated by PLC-δ₁. In contrast, IP formation in response to the activation of the 5-HT₂AR receptor is dependent upon Gₛq₁₁ 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 IP₃ 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 AT₁R activation. However, depletion of RalB has no effect on AT₁R signaling. We found that depletion of RalGDS also inhibits IP production of agonist-stimulated AT₁R. 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 AT₁R 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-AT₁R-stimulated IP formation. A, representative confocal micrographs of HEK293 cells transiently transfected with 5 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT₁R and 1 μg of pcGFP-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-AT₁R and treated with 100 pmol of RalGDS-specific siRNA. D, FLAG-AT₁R-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-AT₁R and treated with 100 pmol of RalGDS-specific siRNA. Data represent the mean ± S.E.M. of four independent experiments. E, FLAG-AT₁R-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 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-AT₁R and treated with 3 mg of pcDNA3.1 plasmid cDNA encoding FLAG-AT₁R and treated with 3 mg of pcDNA3.1 plasmid cDNA encoding β-ArrN1. G, FLAG-AT₁R-stimulated IP formation in response to treatment with 100 nM AngII for 30 min at 37°C in HEK293 cells transfected with 3 mg of pcDNA3.1 plasmid cDNA encoding FLAG-AT₁R. Data represent the mean ± S.E.M. of four independent experiments. *p < 0.05.
AT1R/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, α1B-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 α1B-adrenergic receptors involves the formation of a complex containing Go11 (Park et al., 1998; Feng et al., 1999; Baek et al., 2001). In the present study, we find that the treatment of the AT1R for 30 min with AngII resulted in IP formation that is blocked by both Go11 and PLC-δ1 siRNA treatment. The AT1R stimulates PLC-β-mediated IP formation and the release of Ca2+ from intracellular stores as well as the influx of extracellular calcium.

Fig. 6. Role of Go11 in FLAG-AT1R- and FLAG-5-HT2AR-stimulated IP formation. A, top, immunoblots for endogenous Go11 and actin protein expression in HEK293 cells transfected with 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT1R and treated with 100 pmol of either scrambled or Go11-specific siRNA. Graph shows the effect of Go11-specific siRNA treatment on FLAG-AT1R-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 Go11 and actin protein expression in HEK293 cells transfected with 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-5-HT2AR and treated with 100 pmol of either scrambled or Go11-specific siRNA. Graph shows the effect of Go11-specific siRNA treatment on FLAG-5-HT2AR-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-AT1R-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-AT1R 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-AT1R-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-AT1R, or 2 μg of pcDNA3.1 plasmid cDNA encoding FLAG-AT1R treated with 100 pmol of PLC-δ1-specific siRNA. Cells were treated with CMZ for 10 min before measuring [3H]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 AT1R to PLC-β stimulates IP3-dependent increases in intracellular Ca2+ concentrations that might in part also contribute to the subsequent Ca2+ dependent activation of PLC-δ1 signaling by the AT1R. However, the coupling of the AT1R to PLC-β would be expected to become uncoupled in response to β-agonist binding and the dissociation of the receptor from Gα11. RLGS translocation to the plasma membrane would then lead to the RaLa-dependent release of the PLC-δ1 CaM block, resulting in the activation of IP3 formation via PLC-δ1.

The activation of PLC-δ1 by the AT1R 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; Asemü 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; Asemü et al., 2003). After ischemia, PLC-δ, but not PLC-γ, is degraded by calcium-sensitive proteases (Hwang et al., 2004). This has led to the suggestion that changes in PLC isoyme expression may contribute to alterations in calcium homeostasis in myocardial ischemia. Moreover, PLC-δ1 activation is an important target for tumor necrosis factor receptormediated 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 and the dissociation of the receptor from Gα11, PLCδ1-dependent activation of phospholipase Cγ2 by angiotensin II and epidermal growth factor. J. Biol. Chem 278:49936–49944.


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