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Dual regulation of lysophosphatidic acid (LPA₁) receptor signalling by Ral and GRK

Adel I. Aziziyeh^a, Timothy T. Li^a, Cynthia Pape^a, Macarena Pampillo^{b,c}, Peter Chidiac^a, Fred Possmayer^d, Andy V. Babwah^{a,b,c}, Moshmi Bhattacharya^{a,*}

^a Department of Physiology and Pharmacology, The University of Western Ontario, London, Ontario, Canada N6A 5C1

^b Department of Obstetrics and Gynaecology, The University of Western Ontario, London, Ontario Canada N6A 4V2

^c The Children's Health Research Institute, The University of Western Ontario, London, Ontario Canada N6C 2V5

^d Department of Biochemistry, The University of Western Ontario, London, Ontario, Canada N6A 5C1

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ABSTRACT

Lysophosphatidic acid (LPA) is a major constituent of blood and is involved in a variety of physiological and pathophysiological processes. LPA signals via the ubiquitously expressed G protein-coupled receptors (GPCRs), LPA₁ and LPA₂ that are specific for LPA. However, in large, the molecular mechanisms that regulate the signalling of these receptors are unknown. We show that the small GTPase RalA associates with both LPA₁ and LPA₂ in human embryonic kidney (HEK 293) cells and that stimulation of LPA₁ receptors with LPA triggers the activation of RalA. While RalA was not found to play a role in the endocytosis of LPA receptors, we reveal that LPA₁ receptor stimulation promoted Ral-dependent phospholipase C activity. Furthermore, we found that GRK2 is required for the desensitization of LPA₁ and LPA₂ and have identified a novel interaction between RalA and GRK2, which is promoted by LPA₁ receptor activity. Taken together, these results establish RalA and GRK2 as key regulators of LPA receptor signalling and demonstrate for the first time that LPA₁ activity facilitates the formation of a novel protein complex between these two proteins.

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1. Introduction

The bioactive lipid molecule lysophosphatidic acid (LPA) is a known mediator of diverse biological processes including cell survival, cell differentiation and proliferation, gene transcription, olfaction, as well as cardiovascular function, reproduction and brain development. LPA also mediates cell migration that is required for processes such as wound healing, tissue remodelling, and neurogenesis [1]. A role for LPA has also been implicated in various pathophysiological processes such as cancer, myocardial injury, hypertension, atherosclerosis, and neurodegenerative and psychiatric diseases [2-8]. LPA is detected in blood, and in humans, LPA concentration in serum is high (1–5 uM). with major sources being activated platelets, fibroblasts and adipocytes [1]. LPA evokes its multiple effects through G protein-coupled receptors (GPCRs) that belong to the endothelial differentiation gene (Edg) family. LPA₁/Edg2, LPA₂/Edg4 and LPA₃/Edg7, are specific, highaffinity receptors for LPA and share approximately 50% sequence identity [7,9]. LPA₁ and LPA₂ are ubiquitously expressed, whereas LPA₃ is highly expressed in the brain, lung and ovary. Four other structurally distinct LPA receptors, LPA_{4–7} have been recognized that belong to the P2Y family of nucleotide receptors, but less is known about these receptors [10]. LPA₁ and LPA₂ activate diverse signalling pathways by coupling to multiple heterotrimeric G proteins, namely G_q to activate the enzyme phospholipase C (PLC), G_i and G_s to modulate adenylate cyclase activity, and G_{12/13} activating Rho GTPases [11].

LPA activity is believed to be regulated at the level of receptor desensitization, internalization or sequestration, and degradation. To date, very little has been directly reported on these early regulatory events at the molecular level. Homologous desensitization of GPCRs is regulated by G protein-coupled receptor kinases (GRKs) that constitute a family of seven serine/threonine protein kinases. GRKs exclusively phosphorylate agonist-occupied GPCRs leading to the uncoupling the receptor from heterotrimeric G proteins [12,13]. Thus far, only one study has implicated the ubiquitously expressed GRK2 in the desensitization of endogenous LPA receptors in rat thyroid cells, although it is unknown which specific LPA receptor(s) were desensitized [14]. While LPA₁ has been shown to internalize via β -arrestin- and clathrin-dependent pathways [15,16], to date there are no published studies on the regulation of LPA₂.

Several studies have implicated a role for the small GTP-binding protein Ral in receptor-mediated endocytosis (reviewed, [13,17]). Ral GTPases are members of the Ras superfamily and consist of two isoforms, RalA and RalB, which share 85% sequence identity [18]. Ral is primarily localized to the plasma membrane but is also observed in endocytic vesicles [18]. In addition to its role in cell transformation,

Abbreviations: LPA, lysophosphatidic acid; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; PTX, 293 cells, pertussis toxin; MEFs, mouse embryonic fibroblasts; IP3, inositol 1,4,5-trisphosphate; ARF, ADPribosylation factor; Edg, endothelial differentiation gene; PLC, phospholipase C; RalBP1, Ral-binding protein 1.

^{*} Corresponding author. Tel.: +1 519 661 2111x82970; fax: +1 519 661 3827. E-mail address: Moshmi.Bhattacharya@schulich.uwo.ca (M. Bhattacharya).

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differentiation, and gene transcription, Ral also regulates the cytoskeleton, cell migration, and cell adhesion [18,19]. In addition, Ral can regulate the endocytosis of certain GPCRs such as the metabotropic glutamate receptors (mGluR1a and mGluR5) [20], as well as tyrosine kinase receptors epidermal growth factor and insulin receptors, acting via its downstream effectors phospholipase D and Ral-binding protein 1 (RalBP1) [21–23]. However, a role for Ral in GPCR function is largely unknown. This study reports for the first time that RalA interacts with both LPA₁ and LPA₂. Although RalA does not appear to be necessary for LPA receptor endocytosis, this small GTPase critically regulates LPAinduced inositol phosphate formation. Furthermore, we report a novel interaction between RalA and GRK2 in response to LPA activation, and that these proteins determine the outcome of LPA receptor activation.

2. Materials and methods

2.1. Materials

The HEK 293 cell line was purchased from ATCC (Manassas, VA). Lysophosphatidic acid was purchased from Avanti Polar Lipids (Albaster, AL). Protein G-Sepharose beads were from Amersham Biosciences (Oakville, Ontario, Canada). The anti-Flag M2 agarose affinity beads, polycolonal antibodies and all other biochemical reagents were purchased from Sigma (St. Louis, MO). Flag-tagged LPA₁ and LPA₂ receptors were obtained from Dr. G. Mills (MD Anderson Cancer Institute, Houston, TX).

2.2. Cell culture and transfections

HEK 293 cells were grown at 37 °C in Eagle's minimum essential media (MEM; Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum and 50 μ g/ml gentamicin. Cells were transiently transfected with various plasmid cDNAs using a modified calcium phosphate method [19]. Fresh media was added to the cells 18 h after transfections and the cells were allowed to recover for 8 h before being reseeded onto either 12-well plates for receptor internalization assays, 24-well plates for inositol phosphate production assays, or 35-mm glass-bottomed culture plates for confocal microscopy.

2.3. Gene knockdowns

Gene knockdown of RalA and RalB in HEK 293 cells was achieved using shRNA constructs (OriGene Technologies, Rockville, MD) and introduced into cells by electroporation (Gene Pulser Xcell; Bio-Rad, Mississauga, Ontario, Canada) according to manufacturer's instructions. A heterogeneous population of stable transfectants was selected by using media containing 1 μ g/ml puromycin. Knockdown of each gene was examined using four different shRNA constructs and stable HEK 293 cell lines generated to express each shRNA construct individually. Knockdown of each target gene was verified by Western blot analysis for the corresponding proteins and the two constructs that decreased gene expression the most were utilized for subsequent experiments. Sequences for shRNA constructs are as follows:

RalA shRNA #5: GATGAGAATGTTCCATTTCTACTGGTTGG RalA shRNA #6: CTGGTTGGTAACAAATCAGATTTAGAAGA RalB shRNA #7: CTAGAGGAGCGGAGGCAGGTGCCTGTGGA RalB shRNA #8: GGACAAGGTGTTCTTTGACCTAATGAGAG

2.4. Inositol phosphate formation

Inositol phosphates were radiolabelled by incubating the cells overnight with 1 μ Ci/ml [³H]myo-inositol in Dulbecco's modified Eagle's medium (DMEM; Sigma). Un-incorporated [³H]myo-inositol was removed by washing the cells with Hank's buffered salt solution (HBSS; 116 mM NaCl, 20 mM HEPES, 11 mM glucose, 5 mM NaHCO₃, 4.7 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.2 mM KH₂PO₄, pH 7.4). The cells were pre-incubated for 1 h in HBSS at 37 °C and then preincubated in 500 µl of the same buffer containing 10 mM LiCl for an additional 10 min at 37 °C. The cells were then incubated in either the absence or the presence of 10 µM LPA for 1 h at 37 °C. For pertussis toxin (PTX) pre-treatments, cells were incubated in serum-free medium containing 300 ng/ml PTX for 1 h prior to stimulation. The reaction was stopped on ice by adding 500 µl of 0.5 M perchloric acid and then neutralizing with 400 µl of 0.72 M KOH and 0.6 M KHCO₃. The total [³H]inositol incorporated into the cells was determined by counting the radioactivity present in 50 µl of the cell lysate. Total inositol phosphate was purified from the cell extracts by anion exchange chromatography using AG 1-X8 strong anion exchange resin (Bio-Rad). [³H]Inositol phosphate formation was determined by liquid scintillation using a 1214 RackBeta liquid scintillation counter (LKB Wallac). Total inositol phosphate formed from unstimulated HEK 293 cells transfected with the various constructs were used as a baseline.

2.5. Co-immunoprecipitation and Western blot analysis

HEK 293 cells from 100-mm dishes, transiently transfected with the various cDNA constructs, were treated in either the absence or presence of 10 µM LPA for the indicated times at 37 °C. The cells were then washed once with ice-cold phosphate-buffered saline (PBS) and were lysed in 500 µl of cold lysis buffer (25 mM Hepes, 300 mM NaCl, 1.5 mM MgCl₂, 0.1% Triton X-100) with protease inhibitors ($20 \mu g/ml$ leupeptin, 20 µg/ml aprotinin, and 20 µg/ml AEBSF). 500 µg of total cell lysate proteins was used. Flag-LPA1 and Flag-LPA2 were immunoprecipitated using polyclonal anti-Flag antibody (Rabbit) and protein G-Sepharose beads or using anti-Flag M2 agarose affinity beads for 12-16 h at 4 °C. The beads were washed with lysis buffer, solubilized in SDS sample buffer, and separated by SDS-PAGE. The proteins were transferred onto nitrocellulose membranes by semidry electroblotting. The membranes were blocked with 10% skim milk in TBS-T wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, 0.05% Tween 20) and then incubated with rabbit anti-GFP (Molecular Probes, Eugene, OR), mouse anti-myc (Millipore, Billerica, Massachusetts) or mouse anti-RalA (BD Transduction, Mississauga, Ontario, Canada) antibodies diluted 1:1000 in TBS-T containing 3% skim milk. The membranes were rinsed with TBS-T and then incubated with secondary horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG (Amersham Biosciences) diluted 1:2500 in TBS-T containing 3% skim milk. Membranes were rinsed with TBS-T and were incubated with ECL Western blotting detection reagents (Fisher, Nepean, Ontario, Canada). Densitometric analysis was done using VersaDoc Imaging System (Bio-Rad).

2.6. Single turnover receptor internalization assays

Flow cytometry was used to measure the internalization of Flag–LPA₁ and Flag–LPA₂ as described previously [20]. Cell surface epitope-tagged receptors were prelabeled with primary anti-Flag mouse monoclonal antibody (1:750) on ice for 45 min. Cells were then warmed to 37 °C either in the absence or presence of agonist (10 μ M LPA) for the times indicated in the figure legends and the receptors were allowed to internalize. Cells were then transferred back on ice and labelled with secondary FITC-conjugated anti-mouse IgG antibody (1:500 dilution) for 45 min. Under these conditions, receptors are able to undergo only a single round of internalization. Receptor internalization is defined as the fraction of total cell receptors lost from the cell surface and thus is not available for labelling with the secondary antibody.

2.7. Confocal microscopy

Confocal microscopy was performed using an LSM-510 META laser scanning microscope (Zeiss, Oberkochen, Germany) using a Zeiss 63X,



Fig. 1. LPA₁ and LPA₂ associate and colocalize with RalA. A and B) Representative immunoblots showing the co-immunoprecipitation of GFP–RalA with Flag–LPA₁ and Flag–LPA₂. HEK 293 cells transiently expressing A) Flag–LPA₁ or B) Flag–LPA₂ and GFP–RalA were immunoprecipitated using a monoclonal anti-Flag antibody. Cells co-expressing the vector (pFlagA1) and GFP–RalA were used as a control. C) Representative laser scanning confocal micrographs show the subcellular distribution of internalized Flag–LPA₁ receptors labeled with Alexa Fluor 555-conjugated monoclonal Flag antibody (red) and GFP–RalA (green). HEK 293 cells were transiently transfected with Flag–LPA₁ receptors and GFP–RalA and stimulated with 10 µM LPA for 10 min. Scale bar, 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

numerical aperture 1.4, oil immersion lens and filters with emission wavelengths of 488 and 514 nm. HEK 293 cells expressing Flag–LPA₁ or Flag–LPA₂ with GFP–RalA or PLD2–YFP were plated on 35-mm glass-bottomed culture dishes for live-cell imaging. Anti-Flag mono-clonal antibody was conjugated to Alexa Fluor 555 using the Zenon Mouse IgG Labeling kit (Molecular Probes), following the manufacturer's instructions, just before confocal imaging.

2.8. Statistical analysis

One-way ANOVA tests were used to assess statistical significance with GraphPad StatMate software (GraphPad Software Inc.). Inositol phosphates dose responses data were analyzed by nonlinear regression using a sigmoidal curve fit with a variable slope. The presented EC_{50} and maximal responses are the means obtained from the individual fits to multiple independent data sets. Student's *t* test was used to assess the data (GraphPad Software Inc.). *p*<0.05 was considered statistically significant.

3. Results

3.1. RalA interacts and colocalizes with LPA₁ and LPA₂ receptors

The small Ras-like G proteins of the Ral subfamily are activated downstream of certain receptor tyrosine kinases and GPCRs [24] and can play a role in receptor endocytosis [20]. Previously, for example, it was shown that metabotropic mGLUR1 glutamate receptors can activate RalA, which in turn promotes phospholipase D-dependent internalization of this GPCR [20]. We found that both Flag-LPA₁ and Flag-LPA₂ could constitutively associate with GFP-RalA when coexpressed in HEK 293 cells by co-immunoprecipitation experiments (Fig. 1A and B). To further study the interaction between RalA and LPA₁, we examined their intracellular distribution patterns before and after treatment with the agonist LPA by immunofluorescence and visualization by live-cell confocal microscopy. Prior to LPA treatment, both proteins were found predominantly at the cell surface (Fig. 1C, upper panels). The addition of agonist led to the internalization of both proteins into endocytic vesicles, with observed colocalization between internalized Flag-LPA1 and GFP-RalA as indicated by yellow in the overlay (Fig. 1C, lower panels). Furthermore, Flag–LPA₁ was found to colocalize with endosomal marker proteins GFP-Rab5 (data not shown) as previously reported [15]. Similar results were obtained in cells expressing Flag-LPA₂ (in place of LPA₁), with the addition of 10 μ M LPA once again resulting in receptor internalization into endocytic vesicles that were positive for GFP-RalA (data not shown). Taken together, these results suggest that RalA may regulate LPA1 and LPA₂ receptor signalling and endocytosis.

3.2. RalA is activated by LPA

We next sought to determine whether the stimulation of cells with LPA could activate Ral proteins. The activation of endogenous RalA was assessed in serum-starved HEK 293 cells through its binding to RalBP1, an effector protein which is selective for the GTP-bound form of RalA. We



Fig. 2. LPA stimulates the activation of RalA. Representative immunoblots showing the activation of RalA in response to A) LPA₁ and B) LPA₂ receptor activation. HEK 293 cells transiently expressing Flag–LPA₁ or Flag–LPA₂ were stimulated with 10 μ M LPA for specified times and lysates extracted. Activated GTP-bound Ral (Ral–GTP) was pulled down using RalBP1 beads and immunoblotted for RalA. Total RalA and β -actin expression was examined to control for equal loading of samples. Densitometric analysis shows the mean \pm SE of activated RalA in response to agonist stimulation compared to constitutive activation (0 min). Data shown are representative of three independent experiments. *p<0.05 as compared to time zero.

observed a significant stimulation of RalA activity in cells expressing LPA₁ at 7.5 min of LPA treatment, as compared to unstimulated cells (Fig. 2A). In cells expressing LPA₂, we observed high basal RalA activity in the absence of agonist, and the levels of GTP–Ral did not change significantly upon stimulation with LPA (Fig. 2B). In parallel assays with non-transfected cells, recovery of activated RalA was negligible in both the presence and absence of LPA (data not shown). It follows that this small G protein is activated in an agonist-dependent manner by LPA₁ and in an agonist-independent manner by LPA₂.

3.3. RalA does not play a role in regulating LPA₁ and LPA₂ endocytosis

The data shown in Fig. 1 indicate that Ral interacts with LPA₁ and LPA₂ and appears to co-internalize with both receptors in endocytic vesicles. Since Ral GTPases promote the endocytosis of other receptors [20–23], we investigated whether RalA activity might be required for the endocytosis of LPA₁ and LPA₂. Thus we tested the effect of overexpressing wild-type RalA (RalA WT), GTP hydrolysis-deficient mutant (RalA G23V), or a GTP-binding defective mutant (RalA S28N) on the agonist-dependent loss of cell surface Flag–LPA₁ and Flag–LPA₂ in HEK 293 cells. These mutants have been shown to inhibit the internalization of EGF and mGluR receptors [14,25]. We found that the endocytosis of both Flag–LPA₁ and Flag–LPA₂ was essentially unaffected by the expression of either RalA or its mutants

(Fig. 3A-i and A-ii). We also observed that the extent of agonist-induced internalization of LPA_2 is higher than that of LPA_1 . Therefore, although we have shown that RalA interacts and co-internalizes with LPA_1 and LPA_2 , this small G protein does not appear to play a role in their endocytosis.

To verify the apparent insensitivity of LPA₁ and LPA₂ endocytosis to Ral, HEK 293 cells stably expressing shRNA against RalA, or RalB were generated (Fig. 3B) and tested (Fig. 3C). Stable expression of each construct significantly reduced the expression of its respective target isoform at the protein level, as determined by Western blot analysis (Fig. 3B). These knockdown effects were isoform-specific, as RalA shRNA had no effect on RalB protein levels nor did RalB shRNA affect RalA levels (data not shown), while the expression of a scrambled sequence had no effect on the expression of either Ral protein (Fig. 3B). We found that knocking down RalA or RalB gene expression has no effect on either LPA1 or LPA2 endocytosis using flow cytometry (Fig. 3C-i and C-ii), reinforcing the notion that the agonist-induced internalization of these receptors is not dependent on Ral protein expression. The present results stand in contrast to previous findings showing that Ral plays a role in mGluR1 internalization, notwithstanding that LPA and mGluR receptors similarly bind constitutively to Ral and that both promote Ral activation in response to agonist treatment. This suggests that Ral activation by these two receptor types may lead to the activation of different downstream events.



Fig. 3. Ral does not play a role in endocytosis of LPA₁ and LPA₂. A) Time courses for the agonist-stimulated internalization of i) Flag-LPA₁ and ii) Flag-LPA₂ in the absence (control) or presence of RalA WT, RalA G23V, or RalA S28N. Flow cytometry was used to measure the internalization as a percentage loss of cell surface receptors from the plasma membrane. HEK 293 cells transiently expressing Flag-LPA₁ or Flag-LPA₂ and RalA WT, RalA G23V or RalA S28N were stimulated with 10 µM LPA for the indicated times. B) Representative immunoblots showing knockdown of i) RalA and ii) RalB in HEK 293 cells stably expressing shRNA for RalA and RalB genes, respectively. HEK 293 cells stably transfected with a scrambled sequence were used as a control. Densitometric analysis showed a significant knockdown compared to control. Down regulation of RalA and RalB agenes the agonist-stimulated internalization of i) Flag-LPA₁ and ii) Flag-LPA₂ in HEK 293 cells. C) Time courses for the agonist-stimulated internalization of i) Flag-LPA₁ and iii) Flag-LPA₂ in HEK 293 cells or in HEK 293 cells stably expressing a scrambled sequence were used as a control. Densitometric analysis showed a significant knockdown compared to control. Down regulation of RalA and RalB was achieved using shRNA sequences stably transfected in HEK 293 cells. C) Time courses for the agonist-stimulated internalization of i) Flag-LPA₁ and ii) Flag-LPA₂ in HEK 293 parent cells or in HEK 293 cells stably expressing a scrambled sequence or Ral shRNA constructs. Flow cytometry was used to measure the internalization as a percentage loss of cell surface receptors from the plasma membrane. LPA receptors were stimulated with 10 µM LPA for the specified times. HEK 293 cells were transiently transfected with Flag-LPA₂. The data represent the means ± SE of three independent experiments.

3.4. Ral promotes LPA₁ and LPA₂ receptor-dependent PLC activation

The failure of Ral GTPases to promote LPA₁ and LPA₂ endocytosis suggests that they might regulate other aspects of receptor signalling. Intriguingly, two recent studies [26,27] have shown that phospholipase C (PLC) can be stimulated by activated Ral. This is consistent with findings that other small GTPases, such as Ras, Rac, and Rho, can directly activate various members of this enzyme family [28]. Both LPA₁ and LPA₂ are known to stimulate phosphoinositide (PI) hydrolysis formation via PLC, thereby producing the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP₃). These LPA receptor subtypes are known to signal via G_i, G_q and G_{12/13}, and thus could potentially stimulate PLC β isoforms via either via G_i, or G_q.

To characterize the activation of PLC by LPA₁ and LPA₂, we first generated dose response curves for inositol phosphate production in response to LPA. Total inositol phosphates (IP) were radiolabelled by incubating cells expressing LPA₁ or LPA₂ with [³H]-myo-inositol. Total [³H]-myo-inositol incorporated into cells was measured in 50 µl of cell lysates and total IP formation was purified by anion exchange chromatography. Cells were stimulated with a range of LPA concentrations (0.01 μ M–10 μ M) for 1 h at 37 °C. The EC₅₀ for LPA₁ was 2.3 μ M, while for LPA₂ the EC₅₀ was 0.1 μ M (Fig. 4A-i and A-ii). Further experiments were carried out at 10 μ M LPA (as previously used by others [25,29]) to ensure that receptors were fully activated. To determine the duration of stimulation required for IP production, receptors were stimulated for up to 90 min, and for the first hour yielded linear increases over time in IP



Fig. 4. Dose and time response for LPA₁ and LPA₂ receptor induced inositol 1,4,5-trisphosphate formation. A) HEK 293 cells transiently transfected with either i) Flag–LPA₁ or ii) Flag–LPA₂ were treated with increasing concentrations of LPA (0.01 μ M–10 μ M) for 1 h at 37 °C. B) HEK 293 cells transiently transfected with either i) Flag–LPA₁ or ii) Flag–LPA₂ stimulated with 10 μ M LPA for the indicated times in graphs. HEK 293 cells were transiently transfected with 5 μ g of Flag–LPA₁ or 2.5 μ g of Flag–LPA₂ plasmid cDNA. C) HEK 293 cells transiently transfected with LPA₁ or LPA₂ were stimulated with 10 μ M LPA at 37 °C for 1 h. Cells were either incubated in the presence or absence of 300 ng/ml PTX for 1 h during serum starvation immediately prior to the assay. The data points represent the means \pm SE of triplicate samples from three independent experiments.

production for both receptors, and hence a sixty-minute incubation was used for subsequent experiments (Fig. 4B). Pre-treatment of cells with pertussis toxin (PTX) did not affect LPA-induced IP formation in response to stimulation of LPA₁ or LPA₂ (Fig. 4C), suggesting that the observed activation of PLC is G_i-independent.

To study the potential role of RalA in regulating LPA-stimulated IP production, we expressed LPA₁ or LPA₂ either alone or together with wild-type (RalA WT) or dominant negative RalA (RalA S28N). Dose response curves revealed that RalA WT enhanced LPA-induced IP formation in cells expressing LPA₁ compared to cells expressing receptor alone, whereas the co-expression of RalA S28N attenuated LPA-induced IP formation (Fig. 5A-i). In order to further validate a role for Ral in LPA₁-mediated signalling, we tested the effect of knocking down endogenous RalA in cells expressing LPA₁. Depleting RalA

attenuated LPA-induced IP production compared to cells expressing the receptor and scrambled shRNA (Fig. 5B-i). In contrast to LPA₁, the expression of RalA WT or RalA S28N had little or no effect on LPAinduced IP production via LPA₂ (Fig. 5A-ii). Consistent with these observations, the loss of RalA expression had no significant effect on IP production compared to cells expressing LPA₂ and scrambled shRNA (Fig. 5B-ii).

Overall our data indicate that LPA₁ stimulates PLC activity in a manner that is dependent on Ral activation, whereas PLC stimulation via LPA₂ is not. This difference between receptors is consistent with the observed ability of LPA to stimulate Ral activation via LPA₁ but not LPA₂ (Fig. 2), and it thus appears that the two receptor subtypes promote PLC activity via distinct mechanisms. While PLC_β can be activated by both G_q [30] and Ral [26], it is possible that the observed



Fig. 5. RalA regulates LPA₁ and LPA₂ receptor-mediated signalling. A) LPA dose responses of i) Flag–LPA₁ and ii) Flag–LPA₂ receptor-stimulated IP formation in HEK 293 cells coexpressed with a vector control, RalA WT or RalA S28N cDNAs. HEK 293 cells were transiently transfected with 5 µg of Flag–LPA₁ or 2.5 µg of Flag–LPA₂ receptor plasmid cDNA. 5 µg of plasmid cDNA encoding a vector, RalA WT or RalA S28N were co-transfected. Cells were stimulated for 60 min with increasing doses of LPA. B) LPA dose response of i) Flag–LPA₁ and ii) Flag–LPA₂ receptor-stimulated IP formation in parental HEK 293 cells and in HEK 293 cells stably expressing RalA shRNA. HEK 293 cells were transfected with plasmids encoding 5 µg of Flag–LPA₁ or 2.5 µg of Flag–LPA₂ plasmid cDNA. The data points represent the means ± SE of triplicate samples from three independent experiments.

responses to LPA₁ activation may involve other Ral-sensitive PLC isoforms such as PLC δ [27] and PLC ϵ [26].

3.5. GRK2 desensitizes LPA_1 and LPA_2 in a phosphorylation-independent manner

The molecular mechanisms of desensitization of LPA₁ are largely unknown, and there appear to be no published reports describing the mechanism of LPA₂ regulation. In spite of the observed co-internalization, the present results clearly do not implicate RalA in either LPA₁ or LPA₂ desensitization, thus suggesting other mechanisms. Previous studies have shown that GRK2 desensitizes endogenous LPA receptors in rat thyroid cells but it was not known specifically as to which LPA receptor subtype(s) it desensitizes in those cells [14]. We examined whether GRK2 contributes to the attenuation of LPA1 and/or LPA2 activity due to prolonged agonist exposure. Co-immunoprecipitation experiments demonstrate that GRK2 binds both LPA1 and LPA2 in the absence of agonist (Fig. 6A-i and A-ii). To investigate the role of GRK2 in LPA1 and LPA2 receptor desensitization, we co-expressed GRK2 WT (wild-type), GRK2 CT (GRK2 mutant that inhibits interaction of GRK2 with the receptor) or GRK2 K220R (kinase-deficient mutant) with LPA1 or LPA₂ receptors. Inositol phosphate production was measured in the absence and presence of increasing concentrations of LPA for 1 h at 37 °C. The expression of LPA₁ or LPA₂ was verified for each assay using flow cytometry (data not shown). We found that the expression of either GRK2 WT or GRK2 K220R attenuated LPA-induced IP formation when co-expressed with either LPA1 (Fig. 6B-i) or LPA2 (Fig. 6B-ii) as compared to expression of a vector control. In contrast, GRK2 CT had little or no effect on IP formation when co-expressed with either LPA₁ or LPA₂ receptor (Fig. 6B-i and B-ii). Taken together, these results indicate that GRK2 interacts with both LPA₁ and LPA₂ receptors, and this interaction promotes receptor desensitization in a manner that does not require kinase activity.

3.6. RalA and GRK2 mutually inhibit each other's binding to LPA1 receptors

Since RalA positively regulates signalling of LPA₁, whereas GRK2 attenuates signalling, we hypothesized that the inhibitory effect of GRK2 might reflect an ability to interfere with receptor-RalA coupling. Consistent with this possibility, we found that co-expressing increasing levels of GRK2 together with LPA₁ receptors and RalA significantly decreased the ability of the latter two proteins to co-immunoprecipitate (Fig. 7A). Similar results were obtained when conversely increasing concentrations of GFP-RalA led to a decreased yield of GRK2 that co-immunoprecipitated with the receptor (Fig. 7B). These results suggest that RalA and GRK2 may bind to overlapping sites on LPA₁, or alternatively that there may be negative allosterism between these two receptor-binding proteins. Regardless of how this mutual inhibitory effect occurs, the decreased binding of RalA to the receptor due to GRK2 might be expected to interfere with the ability of LPA to promote Ral activation, thereby potentially having an inhibitory effect on agonist- and RalA-dependent phospholipase C activation by the receptor.

3.7. Agonist-dependent dissociation of RalA and GRK2 from LPA₁ receptors and RalA–GRK2 complex formation

To further study the apparent three-way interaction between LPA₁, RalA, and GRK2, we examined the effects of agonist treatment on the ability of these proteins to co-immunoprecipitate. As noted above, in



Fig. 6. LPA₁ and LPA₂ receptors associate with GRK2. A) Representative immunoblots showing the co-immunoprecipitation of GRK2–myc with i) Flag–LPA₁ and ii) Flag–LPA₂. Lysates from serum-starved HEK 293 cells transiently expressing Flag–LPA₁ or Flag–LPA₂ and GRK2–myc were immunoprecipitated using a monoclonal anti-Flag antibody. B) Effect of GRK2 and its mutants on agonist-stimulated LPA₁ and LPA₂ receptor-stimulated inositol phosphate formation in HEK 293 cells. LPA dose responses of i) LPA₁ and ii) LPA₂ receptor-stimulated IP formation in HEK 293 cells. CPA dose responses of i) LPA₁ and ii) LPA₂ receptor-stimulated IP formation in HEK 293 cells. LPA dose responses of i) LPA₁ and ii) LPA₂ receptor-stimulated IP formation in HEK 293 cells co-expressed with vector (control), GRK2 WT, GRK2 CT, and GRK2 K220R cDNAs. HEK 293 cells were transiently transfected with 5 µg of Flag–LPA₁ or 2.5 µg of Flag–LPA₂ receptor plasmid cDNA encoding a vector, GRK2-myc, GRK2 WT, GRK2 CT, or GRK2 K220R were co-transfected. Cells were stimulated for 60 min with increasing doses of LPA. The data points represent the means ± SE of triplicate samples from three independent experiments.

the absence of agonist stimulation, the LPA₁ receptor bound to RalA when the two proteins were co-expressed (Fig. 8A), and similarly the receptor co-immunoprecipitated with GRK2 when co-expressed with it (Fig. 8B). The addition of 10 μ M LPA decreased the yield of LPA₁-RalA co-immunoprecipitation (Fig. 8A), and similarly LPA₁-GRK2 association also was decreased upon agonist treatment (Fig. 8B). While the time courses of these two agonist effects are not identical, it may be noted that in both cases co-immunoprecipitation was substantially reduced 7.5 min after the addition of agonist.

We also assayed the effects of agonist treatment when all three proteins were expressed together, i.e., GFP–RalA, Flag–LPA₁, and GRK2–myc. Surprisingly, 7.5 min after the addition of LPA we observed the formation of an apparent RalA–GRK2 complex (Fig. 8C). The temporal convergence between this effect and the agonist-dependent decreases in the co-immunoprecipitation of each of these proteins with the receptor suggests that the complex forms concurrently with or subsequent to the dissociation of RalA and GRK2 from the receptor (although based on the present data we cannot formally rule out the possibility that the RalA–GRK2 complex may also include the receptor, as agonist-promoted dissociation of these proteins to the receptor may not be complete).

One conceivable explanation for the observed agonist-dependent decrease in receptor–GRK2 co-immunoprecipitation could be that the conformational change that corresponds to receptor activation decreases the affinity of GRK2 for the receptor; however this explanation contradicts the well established tendency for GRK2 to bind with *higher* affinity to activated GPCRs, although whether such holds for LPA₁ is not known. A more likely possibility may be that the agonist-dependent binding of GTP to RalA (the timing of which corresponds well to the various agonist-dependent changes in co-

immunoprecipitation) somehow alters one or more protein–protein interactions and thus promotes dissociation and/or RalA–GRK2 complex formation. Overall, the mechanisms underlying the observed agonist-dependent changes in the binding interactions between these three signalling proteins remain to be determined, as does the effect of GRK2 binding on RalA's ability to signal to phospholipase C and other downstream effectors.

4. Discussion

The present results reveal several novel and unexpected properties of LPA1 and LPA2 function. Our findings show that the small GTPase RalA binds to both receptors and that LPA triggers RalA activation through LPA₁. While Ral GTPases are known to be activated in response to GPCR stimulation, and evidence exists that activated Ral can promote phospholipase C function [26,27], to our knowledge the present study is the first to show Ral-dependent phospholipase C stimulation in response to the activation of a GPCR. In addition, we have uncovered a novel interaction between RalA and GRK2 which is promoted by the activation of LPA1 and corresponds temporally to agonist-promoted RalA activation as well as the apparent dissociation of both RalA and GRK2 from the receptor. Finally, we have shown that the agonist-stimulated endocytosis of both the LPA1 and LPA2 receptors is independent of RalA, but that receptor desensitization can be mediated via GRK2 in a manner that is not dependent on the kinase activity of the latter. This work thus advances our understanding of LPA signalling and the attenuation thereof.

The biologically-active ligand LPA acts as an extracellular signalling molecule and is capable of exerting dramatic effects on a wide variety of



Fig. 7. RalA and GRK2 inhibit each others' binding to LPA₁ receptors. A) Representative immunoblots demonstrating the inhibitory effects of increasing concentrations of GRK2 on RalA binding to LPA₁. GFP–RalA plasmid transfected was kept constant at 5 μ g and amounts of GRK2 plasmid are as indicated. Cells were lysed after 7.5 min of LPA₁ receptor stimulation with 10 μ M LPA. Densitometric analysis shows the means \pm SE of the co-immunoprecipitation between LPA₁ receptors and GRK2 in the presence of of increasing DNA concentrations of GRK2 (0 μ g). B) Representative immunoblots demonstrating the inhibitory effects of increasing concentrations of RalA on GRK2 binding to LPA₁, GRK2 plasmid transfected was kept constant at 5 μ g and amounts of RalA plasmid are as indicated. Cells were lysed after 7.5 min of LPA₁ receptor simulation with 10 μ M LPA. Densitometric analysis shows the means \pm SE of the co-immunoprecipitation between LPA₁ receptors and GRK2 in the presence of GRK2 (0 μ g). B) Representative immunoblots demonstrating the inhibitory effects of increasing concentrations of RalA on GRK2 binding to LPA₁, GRK2 plasmid transfected was kept constant at 5 μ g and amounts of RalA plasmid are as indicated. Cells were lysed after 7.5 min of LPA₁ receptor stimulation with 10 μ M LPA. Densitometric analysis shows the means \pm SE of the co-immunoprecipitation between LPA₁ receptors and GRK2 in the presence of increasing DNA concentrations of RalA compared to absence of RalA (0 μ g). The data shown are representative of three independent experiments. *p<0.05.

cell and tissue types. The LPA₁ receptor was the first LPA receptor identified and is present at high levels in lungs, testis, and brain, specifically in oligodendrocytes, and plays a role in myelination [31]. The importance of the LPA₁ gene in mammalian development is evident by the reduced viability of LPA₁-null mutant mice [29]. The activity of this receptor regulates cellular morphological changes such as stress fiber formation, neurite retraction, cell rounding, and cell motility in response to LPA [32,33]. LPA₂ has a more restricted pattern of expression in comparison to LPA₁. The RNA transcripts for these receptors are high in adult human peripheral blood lymphocytes, thymus, spleen, and testis [34]. There is evidence LPA₁ and LPA₂ may have redundant roles in LPAinduced stress fiber formation, as well as in the regulation of various signalling cascades [29]. Mice deficient for LPA₂ have no obvious phenotype and mice deficient for both LPA₁ and LPA₂ display the same abnormalities as LPA1-deficient mice with an increase in the incidence of frontal hematomas and a decrease in survival [29]. There is increasing evidence implicating LPA₂ receptors as the key mediator in cancer progression, and the expression of the LPA₂ receptor is up-regulated in ovarian cancers, invasive ductal carcinoma tissue of breast cancers, colorectal cancers, and thyroid cancers [35-38].

Both LPA₁ and LPA₂ receptors can regulate multiple intracellular signalling pathways, including the activation of phospholipase C, PI3 kinase, MAP kinase, Ras, and Rho, and the inhibition of adenylyl cyclase; these effects are mediated via coupling to $G_{i/o}$, G_s , G_q , and $G_{12/13}$ and

possibly in a G protein-independent manner [1,39,40]. Our findings are consistent with previous studies showing that PLC stimulation by LPA₁ and LPA₂ receptors is PTX-insensitive [41], thus ruling out $G_{i/o}$ and suggesting the involvement of G_a. Such an interpretation, however, does not fully consider other mechanisms known to exist. In addition to the four isoforms of PLCB, which can be activated by heterotrimeric G proteins, there are at least nine other isozymes that are insensitive to this mode of regulation [28]. The phospholipase activity of PLCE, for example, is enhanced through a direct interaction with RhoA-GTP [28], indicating a possible G_{12/13}-based mechanism. As noted above, Ral itself has also been observed to stimulate PLC activity, a possibility supported by the present observations. Sidhu and colleagues [27] found that Ral activates PLCo1 in a calmodulin-sensitive but nucleotide-insensitive manner, while Kelley et al. [26] found PLCE activity to be stimulated by a constitutively activated form of RalA. Intriguingly, the latter study also showed that endogenous LPA receptors in Cos-7 stimulated the activity of transiently expressed PLCE. These findings suggest that the activation of RalA and its release from LPA₁ in response to agonist treatment could lead to the stimulation of a heterotrimeric G protein-insensitive isozyme such as PLC δ or PLC ϵ , but such possibilities require further study.

The present results indicate that the mechanisms via which LPA₁ and LPA₂ activate PLC signalling may not be the same. While LPA₂ was found to constitutively bind to and activate RalA, no further effect appeared to result from the addition of LPA, in contrast to the stimulatory effect that



Fig. 8. RalA associates with GRK2 in response to LPA₁ receptor activation. LPA stimulation alters the association of LPA₁ with A) RalA and B) GRK2. Representative immunoblots demonstrating the co-immunoprecipitation of Flag–LPA₁ with GFP–RalA and GRK2–myc in the presence of unstimulated and stimulated Flag–LPA₁ receptors. Densitometric analyses show the means \pm SE of the association between LPA₁ receptors and GFP–Ral and GRK2–myc in response to LPA stimulation. The data shown are representative of three independent experiments. **p*<0.05. C) Representative immunoblot demonstrating the co-immunoprecipitation of GRK2–myc with GFP–RalA in the presence of unstimulated and stimulated and stimulated and stimulated shown are representative immunoblet demonstrating the co-immunoprecipitation of GRK2–myc with GFP–RalA in the presence of unstimulated and stimulated Flag–LPA₁ receptors. Densitometric analyses show the means \pm SE of the association between RalA and GRK2 in response to agonist stimulation compared to time zero. The data shown are representative of three independent experiments. **p*<0.05 compared to basal association (0 min).

this agonist had on LPA₁. Correspondingly with the LPA₁ receptor, LPAstimulated phospholipase C activity was profoundly affected by RalA overexpression, knockdown, or mutants, whereas these experimental manipulations were virtually without effect on agonist-dependent PLC activation via LPA₂. Thus our findings point to a role for RalA in mediating LPA₁ but not LPA₂ agonist-dependent effects on PLC.

In contrast to our original hypothesis, Ral did not promote LPA₁ or LPA₂ receptor endocytosis in spite of its apparent ability to cointernalize with these receptors upon agonist treatment. Although GRK2 has been shown to desensitize endogenous LPA receptors in rat thyroid cells [14], it has not been established whether or not GRK2 specifically mediates desensitization of LPA₁ and LPA₂. We found that GRK2 attenuates agonist-stimulated LPA1 activity, whereas the coexpression of GRK2 CT, the C-terminal domain of GRK2 which is involved in the targeting of the kinase to the plasma membrane in response to receptor activation, did not affect agonist- and LPA1 and LPA₂ receptor-dependent IP formation [42]. This indicates that GRK2 may be essential for desensitization and signal attenuation. A kinasedeficient mutant of GRK2, GRK2 K220R, also blocked LPA-induced IP formation similar to GRK2 wild-type, indicating that the desensitization of LPA₁ may occur in a phosphorylation-independent manner. Several recent publications have implicated GRKs in phosphorylationindependent desensitization of various GPCRs [43-47]. It is suggested that phosphorylation-independent desensitization occurs as a result of sequestration of G_{α} by protein–protein interactions [48].

Perhaps the most surprising aspect of the present study is the observed agonist- and LPA1-dependent association of RalA with GRK2. The present results do not distinguish between the possibilities that this novel interaction may contribute to LPA₁ receptor signalling, desensitization, or both, and indeed the outcome may depend upon the stoichiometric relationship between RalA and GRK2. Notably, an excess of either one of these proteins inhibited the ability of the other to associate with the immunoprecipitated LPA₁ receptor in extracts from cells expressing all three proteins, and it follows that the observed loss of agonist-dependent PLC activation due to GRK2 overexpression may have been due at least in part to the inability of RalA to access the receptor. In contrast, the constitutive association to the LPA₁ receptor of both RalA and GRK2 was apparently reversed by agonist activation, and this change was accompanied by the formation of a protein complex containing both RalA and GRK2. The signalling properties of this novel RalA-GRK2 complex at present are unknown and are under investigation. The multidomain protein GRK2 could conceivably serve as an anchoring protein to facilitate interaction between RalA and an effector, or alternatively GRK2 could act to sequester Ral away from other proteins. Further studies will be required to reveal how this novel protein complex fits into the signalling pathways initiated by the activation of LPA₁, and perhaps other GPCRs as well.

5. Conclusions

- Ral associates and colocalizes with LPA₁ and LPA₂ but does not play a role in their internalization.
- GRK2 desensitizes LPA₁ and LPA₂ and this appears to occur in a phosphorylation-independent manner.
- Ral mediates the activation of phospholipase C signalling by LPA₁.
- Ral mediates the signalling of LPA₁ by associating with GRK2. Ral and GRK2 compete for binding to LPA₁ and hence affect the signalling properties of the receptor.

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