THE REGULATION OF CELLULAR TRAFFICKING OF THE
HUMAN LYSOPHOSPHATIDIC ACID RECEPTOR 1:
IDENTIFICATION OF THE MOLECULAR DETERMINANTS
REQUIRED FOR RECEPTOR TRAFFICKING

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THE REGULATION OF CELLULAR TRAFFICKING OF THE HUMAN LYSOPHOSPHATIDIC ACID RECEPTOR 1: IDENTIFICATION OF THE MOLECULAR DETERMINANTS REQUIRED FOR RECEPTOR TRAFFICKING

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<tr>
<td>ATX</td>
<td>Autotaxin</td>
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<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>B2AR</td>
<td>(\beta)2-adrenergic receptor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>1,2-diacylglycerol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dyn</td>
<td>Dynamin</td>
</tr>
<tr>
<td>EEA-1</td>
<td>Early endosome autoantigen-1</td>
</tr>
<tr>
<td>EDG</td>
<td>Endothelial differentiation gene</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
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<tr>
<td>GDP</td>
<td>Guanosine 5’–diphosphate</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
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<tr>
<td>GRK</td>
<td>G-Protein receptor kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine 5’–triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid (18:1; 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>LPA1, 2, 3</td>
<td>Lysophosphatidic acid receptors 1, 2, 3</td>
</tr>
<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>LPP</td>
<td>Lipid phosphate phosphohydrolases</td>
</tr>
<tr>
<td>mACHR</td>
<td>Muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacyl glycerol</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated Protein Kinase</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5 bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated Receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1 phosphate</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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SUMMARY

The following thesis research was undertaken to gain a better understanding of the mechanisms that regulate the cellular trafficking and signaling of the endothelial differentiation gene (EDG) family of G-protein coupled receptors LPA₁, LPA₂ and LPA₃. This project specifically focused on the regulation of the trafficking of the LPA₁ Lysophosphatidic acid receptor.

The initial studies undertaken in this project were aimed at understanding the endocytic pathway followed by the LPA₁ receptor. Lysophosphatidic acid (LPA) stimulates heterotrimeric G protein signaling by activating three closely related receptors, termed LPA₁, LPA₂ and LPA₃. In the first part of the project, we show that membrane cholesterol, in addition to promoting LPA₁ signaling, is essential for the association of LPA₁ with β-arrestin, which leads to signal attenuation and clathrin dependent endocytosis of LPA₁. Reduction of clathrin heavy chain expression, using small interfering RNAs, inhibited LPA₁ endocytosis. LPA₁ endocytosis was also inhibited in β-arrestin 1 and 2-null mouse embryo fibroblasts (β-arrestin 1/2 KO MEFs), but was restored upon re-expression of wild-type β-arrestin 2. β-arrestin attenuates LPA signaling as LPA₁-dependent phosphoinositide hydrolysis was significantly elevated in β-arrestin 1/2 KO MEFs and was reduced to wild-type levels upon re-expression of wild-type β-arrestin. Interestingly, extraction of membrane cholesterol with methyl-β-cyclodextrin inhibited LPA₁ signaling, β-arrestin membrane recruitment and LPA₁ endocytosis. Cholesterol repletion restored all of these functions. However, neither the stimulation of
phosphoinositide hydrolysis by the M1 acetylcholine receptor nor its endocytosis was affected by cholesterol extraction. LPA treatment increased the detergent resistance of LPA$_1$ and this was inhibited by cholesterol extraction, suggesting that LPA$_1$ localizes to detergent-resistant membranes upon ligand stimulation. These data indicate that although LPA$_1$ is internalized by clathrin- and β-arrestin dependent endocytosis, membrane cholesterol is critical for LPA$_1$ signaling, membrane recruitment of β-arrestins and LPA$_1$ endocytosis.

The second phase of the project was aimed at elucidating the different structural motifs required for the trafficking and signaling of the LPA$_1$ receptor and helping us gain a more mechanistic view of the processes involved in its regulation. The LPA$_1$ receptor is the most widely expressed and has been shown to be a major regulator of migration of cells expressing it. In the second part of the project we show that agonist-independent internalization of the LPA$_1$ receptor is clathrin adaptor, AP-2 and PKC-dependent and that it requires a distal dileucine motif, whereas agonist-dependent internalization of the LPA$_1$ receptor is β-arrestin and clathrin-dependent and requires a cluster of serine residues in the tail region, which is upstream of the dileucine motif. Exposure to the PKC inhibitor, Bisindolylmaleimide I (Bis I) inhibited both basal and phorbol 12-myristate 13-acetate (PMA)-induced internalization but not LPA-induced internalization. A cluster of serine residues in the tail region of the LPA$_1$ receptor was required for LPA-induced internalization, β-arrestin2 GFP translocation to the plasma membrane and signal desensitization. In contrast, a dileucine motif (IL) was required for both basal and PMA-induced internalization. Interestingly, β-arrestin2 GFP failed to translocate to the plasma membrane upon brief PMA exposure. Additionally, unlike LPA treatment, upon PMA
exposure the LPA₁ receptor internalized in β-arrestin 1/2 KO MEFs suggesting that
PMA-dependent internalization is β-arrestin independent. To identify the alternate
adaptor protein required for agonist-independent internalization, we treated the
LPA₁/HeLa cells with siRNA against AP-2 to reduce endogenous protein expression
levels. Treatment with AP-2 siRNA inhibited basal and PMA-induced internalization and
partially inhibited LPA-induced internalization. These results indicate that agonist-
dependent internalization is β-arrestin dependent and PKC-independent and requires a
cluster of serines in the tail region, whereas agonist-independent internalization is AP-2
and PKC-dependent, β-arrestin independent and requires a dileucine motif in the tail
region.

These studies collectively vastly enhance our understanding of mechanisms that
regulate LPA₁ trafficking and signaling. These studies can also be applied to other G-
protein coupled receptors making the task easier for other scientists to understand this
vast family of receptors.
CHAPTER 1

INTRODUCTION

1.1 G-protein Coupled Receptors

1.1.1 What are GPCRs?

All organisms, unicellular and multicellular, need to perceive their environment for survival. Most organisms achieve this by utilizing proteins that are embedded on their outer membrane region called receptors. These integral membrane receptors can recognize numerous molecules allowing a cell or an organism to perceive different stimuli in its external environment. G-protein coupled receptors (GPCRs) or seven trans-membrane receptors (7TMRs) are the largest family of integral membrane proteins and can respond to a variety of stimuli such as light, odor, pheromones, taste, hormones and neurotransmitters. GPCRs can respond to a variety of molecules ranging from photons, tastants, and odorants to lipids, ions, peptides and amines (1). GPCRs have been found in all eukaryotes including yeast, plants, insects and mammals(2). Due to their ability to recognize numerous molecules and signals, GPCRs regulate a number of physiological processes in complex animals such as inflammation, light and pain perception, chemotaxis, development and neurotransmission (1, 3, 4).

In humans, GPCRs are encoded by over 800 genes and have a characteristic seven trans-membrane structure (5, 6). The typical GPCR has an extracellular N-terminal domain; seven trans-membrane domains connected by three intracellular and
Figure 1 A G-protein Coupled Receptor (GPCR). The typical GPCR has an N-terminal domain, seven transmembrane (TM) domains, 3 exoloops and 3 cytoloops and a C-terminal domain. The GPCR activated by its cognate ligand binds to and activates heterotrimeric G-proteins that have 3 subunits $\alpha$, $\beta$ and $\gamma$. Activation of heterotrimeric G-proteins leads to an exchange of GDP for GTP leading to various downstream signaling cascades in the cell.
three extracellular loops (7). Their complex structure allows for the recognition and interaction with a myriad variety of molecules both extracellular and intracellular.

GPCRs transduce external signals to intracellular second messengers that allow the cells to translate them into messages that are physiologically relevant and to respond accordingly. The process of signal transduction first involves the binding of a ligand to its cognate GPCR. The binding of ligand causes the GPCR to stabilize into an active conformation leading to an interaction with intracellular heterotrimeric GTP binding proteins (G-proteins) (Figure 2). Heterotrimeric G-proteins are intracellular proteins that are bound to GDP in their nascent stage and exchange the GDP for GTP when bound to active GPCRs. The heterotrimeric G-proteins have 3 subunits $\alpha$, $\beta$ and $\gamma$ (Figure 1). Human G proteins are derived from approximate 35 genes, which include 16 genes encoding $\alpha$-subunits, 5 encoding $\beta$-subunits, and 14 encoding $\gamma$-subunits (6).

The $\alpha$ subunit is divided into four classes: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12/13}$, based on their ability to activate different effector proteins (Figure 2). A GPCR can bind to either one or more $G\alpha$ subunits, thus increasing the complexity of a response. Activation of the GPCR leads to the activation of the heterotrimeric G-protein, which now exchanges GTP for GDP on the $\alpha$ subunit. The $\alpha$ and $\beta\gamma$ subunits separate from each other and go on to either negatively or positively regulate downstream effector proteins (Figure 2). The activation of GPCRs not only leads to activation of heterotrimeric G-proteins and its effectors but also leads to negative regulation of G-protein binding, receptor internalization and G-protein independent signaling.
Figure 2. Diversity of G-protein-coupled receptor signaling. A variety of molecules can bind to and activate more than 800 different GPCRs. The major effectors of GPCRs are the heterotrimeric G-proteins that have α, β and γ subunits. The α subunits are of four type’s viz. Gαs, Gαi, Gαq, and Gα12/13. These α subunits can regulate various effector proteins on the plasma membrane, in the cytosol or in the nucleus.
1.1.2 GPCR signaling and trafficking

GPCR signaling can be divided into G-protein-dependent and –independent signaling. G-protein dependent signaling involves the interaction with heterotrimeric G-proteins. As mentioned before, upon activation the four classes of heterotrimeric G-proteins (Gαs, Gαi, Gαq, Gα12/13) can regulate a variety of downstream effectors. Typically Gαs stimulates adenylyl cyclase and increases levels of cAMP leading to activation of cAMP activated protein kinase, PKA. In contrast, Gαi inhibits adenylyl cyclase activity and decreases cAMP levels leading to reduction in active PKA levels. Additionally, Gαi has been shown to activate the small GTPase Rac-1 leading to changes in lamellipodia formation causing changes in the migratory behavior of cells (8). Gαq stimulates Phospholipase C β (PLCβ) which in turn cleaves phosphoinositide-4, 5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol trisphosphate (IP3). IP3 can then activate its cognate receptors on the endoplasmic reticulum (ER) membrane and cause the release of stored Ca2+ ions. DAG then recruits Protein kinase C (PKC) to the plasma membrane and Ca2+ ions bind to and activate PKC (9, 10). Additionally, Gαi has been shown to activate PLCβ, albeit the activation is many magnitudes lower than that of Gαq induced activation (9). Gα12/13 has been shown to stimulate Rho-GTPases that causes a change in actin dynamics of the cell. In addition to these four subfamilies of Gα, Gαo (transducin) is a type of Gα subunit that regulates cGMP phosphodiesterase activity in the light system in the eye. Apart from the α subunit the βγ subunits (Gβγ) can also regulate various downstream effector proteins such as ion channels, lipid kinase PI3Kγ, PLCβ, src Kinases and G-protein receptor kinases (GRKs) (11). The βγ subunits have also been shown to
transactivate the EGF receptor through src kinases leading to Ras-GTPase activation and subsequent MAPK phosphorylation and transcription of nuclear genes (11, 12). Activation of src kinases through βγ subunits can also lead to direct activation of Ras-GTPases and MAPK phosphorylation (11). Although one ligand molecule stimulates one GPCR, the GPCR in turn can activate multiple second messenger molecules causing signal amplification. Therefore these complex interconnected GPCR signaling pathways can regulate many physiological responses such as migration, growth, cell division, inflammation, development and immunity.

In order to maintain physiological responsiveness biological systems constantly need to diminish their initial response to external stimuli. This process is termed as desensitization or signal attenuation or adaptation (7). Signal desensitization mainly involves the impairment of the interaction of receptor with heterotrimeric G-proteins. Desensitization is a multi-step process that includes: 1) phosphorylation of the receptor by kinases, 2) binding of phosphorylated residues by arrestins, 3) endocytosis and 4) recycling of the receptor back to the plasma membrane (resensitization). Desensitization that occurs as a result of exposure to the receptors cognate ligand is termed as homologous desensitization, whereas desensitization that is mediated due to activation of other receptor systems is termed as heterologous desensitization. Homologous desensitization can be mediated by cytosolic kinases known as G-protein receptor kinases (GRKs), whereas heterologous desensitization typically involves GPCR phosphorylation by second messenger dependent kinases, protein kinase A (PKA) and protein kinase C (PKC). Heterologous desensitization probably has a more prolonged and global effect as compared to
homologous desensitization, thus affecting multiple responses. To date seven subfamilies of GRKs have been identified and so far have been shown to regulate desensitization of multiple receptor systems (1). GRKs have been shown to phosphorylate serine or threonine residues on cytosolic domains of GPCRs (13). Second messenger dependent kinases such as PKA and PKC have been shown to phosphorylate GPCRs either through feedback mechanisms or by heterologous desensitization. Additionally, apart from desensitization, PKC phosphorylation has also been implicated in receptor trafficking (14, 15). Desensitization is indeed an important step in the physiology of a cell as failure to desensitize can cause diseases such as retinitis pigmentosa (16) and on the other hand up-regulation of GRK activity can lead to diseases like myocardial ischemia and chronic heart failure (7).

Once a GPCR has been phosphorylated by GRKs after agonist stimulation, a cytosolic protein known as arrestin can now bind to the GPCR and mediate endocytosis (17). GPCR endocytosis is a major mechanism that is employed to control the number of cell surface receptors (1). The internalization and intracellular trafficking of GPCRs is important: 1) for rapidly “turning off” GPCR signaling (desensitization), 2) for recycling internalized receptors back to the cell surface (resensitization), (18, 19) and 3) for long-term receptor down-regulation through degradation in lysosomes (20). The sorting of GPCRs to lysosomes serves the purpose of attenuating signaling over an extended period of time, as a result of prolonged receptor activation. However, extracellular signals can be attenuated rapidly at the cell surface by phosphorylation of certain residues in the GPCR tail, in an endocytosis independent manner (19, 21). As mentioned before, GPCRs at the plasma membrane
are phosphorylated by G-protein Receptor Kinase (GRKs) upon agonist binding (7) or by Protein Kinase C (PKC). These phosphorylated receptors on the cell surface can then interact with certain proteins called β-arrestins that bind to these phosphorylated residues on the C-terminal tail or the third intracellular loops and physically inhibit the interaction of the GPCR with heterotrimeric G-proteins causing signal attenuation (7, 21). This serves the purpose of rapid resensitization of receptors via sorting to various intracellular compartments where membrane bound phosphatases dephosphorylate these receptors. The dephosphorylated receptors are then recycled back to the surface for another round of ligand-induced activation. Furthermore, these β-arrestin proteins can interact with AP2 and clathrin and therefore localize the receptors towards clathrin-coated pits causing receptor internalization (22-24) (Figure 3). In addition to removing receptors from the cell surface, endocytosis effectively removes the ligand from the surface also.

Typically, GPCRs like the β2AR, PAR-1 and M1mAChR utilize clathrin-dependent pathways for internalization (13) (Figure 3). Alternatively, many GPCRs like the ETA<sub>A</sub>, S1P1 and Bradykinin B2 receptors utilize a clathrin-independent pathway (25). The clathrin-dependent pathway is one of the most well characterized pathways of internalization. Clathrin-independent pathways are still not well understood and further research needs to be done in this field. Most clathrin-independent pathways utilized by GPCRs are either cholesterol- or caveolae-dependent or dependent on both.
Figure 3. GPCR trafficking pathways. Upon agonist stimulation GPCRs localize to Clathrin coated pits (CCPs) and internalize into endosomes. After internalization the GPCR can either recycle back to the surface or be degraded in lysosomes.
These GPCRs localize to specialized microdomains in the plasma membrane termed as Lipid rafts or Detergent resistant membranes (DRMs). These lipid rafts/DRMs are characteristically enriched in cholesterol and glycosphingolipids and are sites for signal transduction for various proteins. A subset of lipid rafts are also enriched in a protein called Caveolin, which acts as an alternative endocytic adaptor protein(26, 27). Caveolae were first identified as flask shaped invaginations located at or near the plasma membrane and upon detergent extraction ended up in the same fractions as lipid rafts. Caveolin, like clathrin, forms a coat around membrane invaginations and mediates internalization. Many signaling proteins like the α subunit of heterotrimeric G-proteins, eNOS and src-like tyrosine kinases have been shown to localize to lipid rafts. Association and localization of GPCRs with lipid rafts/DRMs can vary from receptor to receptor. GPCRs like the Endothelin ETₐ, ETₐ, Sphingosine-1 phosphate S1P₁, Chemokine CCR5 and Bradykinin B₂ receptors are enriched in lipid rafts/DRMs in the nascent stage and internalize via a lipid raft pathway (25), whereas certain GPCRs like the Somatostatin SST2 receptor “move into” lipid rafts upon agonist stimulation and internalize via a caveolae/lipid raft pathway (28). Alternatively, GPCRs like the Angiotensin AT1 move into lipid rafts upon agonist stimulation to activate specific signaling events and then again move out of lipid rafts to internalize via CCPs (29). Finally, the β2AR in some cells has been shown to be localized to lipid rafts in the nascent stage but moves out of lipid rafts upon agonist stimulation and internalizes via CCPs (30). Thus each GPCR seems to have its own itinerary probably based upon the location of its interactors or downstream effectors.
Internalization and desensitization are important processes involved in signal attenuation and recycling of receptors back to the surface. As mentioned before, β-arrestins are also important determinants in the process of desensitization and internalization. Another important process involved in recycling of receptors back to the cell surface is called resensitization, which is a crucial step in maintaining physiological responsiveness. β-arrestins have also been shown to play an important role in resensitization (19). Based on the kinetics of β-arrestin association 2 classes of GPCRs that have been defined: 1) the GPCRs that transiently associate with β-arrestin at the plasma membrane and therefore recycle and resensitize rapidly (Class A) and 2) GPCRs that associate with β-arrestins at the plasma membrane and remain associated with it on endosomes, resulting in slow recycling and resensitization (Class B) (31) (Figure 4). It has also been shown that the C-terminal tail of GPCRs dictates this differential association and that this differential trafficking is completely reversed, when the C-terminal tails of two receptors belonging to the 2 classes are switched. It has been shown that specific serine and threonine residues on the C-terminal tail of GPCRs are phosphorylated and that the presence of acidic residues adjacent to these residues increases the probability of phosphorylation. Recent studies have shown an additional role for β-arrestins in modulating signaling responses, specifically for class B receptors. Class B receptors have β-arrestin bound to them on endosomes and the β-arrestins can then interact with signaling molecules like MAP Kinases (ERK) and induce signaling responses while present on endosomes (32). Thus β-arrestins have a multi-faceted role in both GPCR signaling and trafficking. Although most GPCRs utilize β-arrestin as an adaptor protein there are some GPCRs like the PAR-1 receptor do not require β-arrestin for internalization (33).
Figure 4. β-arrestin association with GPCRs regulates internalization and recycling kinetics. Agonist-dependent activation of GPCRs leads to their phosphorylation and binding of β-arrestin. The arrestin-receptor complex is targeted to clathrin-coated pits (CCPs). In the case of Class A receptors, the arrestin-receptor complex is transient and the receptors rapidly recycle back to the PM, whereas for Class B receptors the arrestin-receptor interaction is not transient and arrestin remains bound to receptor on endosomes resulting in slow recycling of receptors back to the PM.
These GPCRs use an alternative adaptor protein, AP-2, for internalization. AP-2 mediated internalization has been shown to regulate both agonist-dependent and independent internalization of GPCRs.

Although the classical view is that GPCRs on the plasma membrane work as single monomers, recent evidence has suggested that many GPCRs can also form both homo- and hetero-dimers (34). Recent evidence suggests that dimerization of GPCRs can have an effect on both signaling and trafficking. Hetero-dimerization has been implicated in the trafficking of GABA<sub>B</sub> receptors from the ER to the plasma membrane. Hetero-dimerization has also been implicated in the ability of certain taste receptors to recognize the sweet stimuli (35). Olfactory receptors have also been shown to require hetero-dimerization for proper expression at the plasma membrane (36). Thus, heterodimerization seems to regulate both signaling and trafficking of GPCRs.

For the past several years evidence for G-protein independent signaling has also emerged (37). Apart from β-arrestins that can act as signaling mediators on endosomes with Class B GPCRs, the role of other proteins have also been implicated in G-protein independent signaling. SH-2 domain containing proteins have been shown to directly interact with GPCRs like the angiotensin AT1 receptor and B2AR mediating downstream signaling events (38-40). Some GPCRs have also been shown to interact with small GTP binding proteins like ARF- and Rho-GTPases (41). The interaction requires a NPXXY motif that is present at the end of the seventh trans-membrane domain. Many GPCRs like the β2AR have also been known to interact with PDZ domain proteins like Na+/H+ exchange regulatory factor (NHERF) and PSD-95 (42) mediating renal Na+/H+ exchange. Some GPCRs contain polyproline sites either on their third-intracellular loops
or cytoplasmic tails. These polyproline sites have been shown to interact with SH3 domain, WW domain and EVH domain proteins (43). The metabotropic glutamate receptors (mGluRs) have been shown to interact with Homer family of EVH domain proteins through polyproline sites on their cytoplasmic tails (44). Additionally, evidence for G-protein independent signaling has emerged from the family of 7TM receptors called as frizzled. Secreted Wnt proteins regulate tissue polarity during development through the frizzled receptor (45). Although, frizzled can interact with G-proteins, most of its action have been documented as being through other proteins such as dishevelled, GSKβ-3 and β-catenin (46). Thus apart from the typical G-proteins that a GPCR can activate, these new protein interactions increase the complexity of the signaling pathways regulated by GPCRs and opens new avenues for GPCR research.

1.1.3 GPCR structural motifs and interactions

Most GPCRs have many sequence motifs in their amino acid sequence that enables binding of different adaptor proteins that recognize these sequences and are either involved in targeting GPCRs to different compartments or in endocytosis and recycling. Many proteins including GPCRs have consensus sequence motifs in their structure that are involved in sorting and trafficking. Sequence motifs that have been shown to be involved in the trafficking of GPCRs include: 1) Tyrosine based motifs (YXXΦ or NPXY), where Φ is a bulky hydrophobic residue (47); 2) di-leucine motifs that have been shown to be involved in cell surface transport, endocytosis and lysosomal targeting (48-50); 3) Serine clusters that are involved in interactions with β-arrestins and regulate post-
endocytic trafficking of GPCRs (19, 31, 51) and 4) the C-terminal type I PDZ ligands (S/T-X-Φ) (52).

Tyrosine based motifs (YXXΦ) were initially described in the regulation of trafficking and sorting of various receptors like the transferrin and mannose phosphate receptors. Apart from endocytosis, YXXΦ motifs have been implicated in lysosomal sorting (47). GPCRs like PAR-1 have been shown to require the YXXΦ motif for internalization as well as targeting to lysosomes for degradation (53). Additionally, tyrosine-based motifs on several GPCRs have been shown to interact with adaptor protein AP-2 and promote clathrin-dependent endocytosis (33, 54). NPXY motifs have also been implicated in the regulation of trafficking of GPCRs like Somatostatin receptor type 5 (SST5) and the CB2 cannabinoid receptor (55-57).

Dileucine based motifs were first described in the regulation of trafficking of the CD3 T cell antigen wherein the regulatory sequence was revealed as DKQTLL, where the dileucines were the most important residues (58). Since then many proteins including GPCRs have been shown to require dileucine-based motifs for their trafficking. Dileucine motifs have been shown to regulate trafficking of GPCRs like CXCR4, V2R and B2AR (48, 59, 60). Dileucine motifs, like tyrosine-based motifs, have been shown to interact with AP-2, to promote internalization (55, 61).

PDZ domains are conserved protein modules that mediate protein–protein interactions. The term “PDZ” is derived from the first letters in the names of the three proteins in which these modules were originally characterized: PSD-95, Dlg, and ZO-1 (62). PDZ domains bind to the C-terminal tails of target proteins, and the binding preferences of a number of PDZ domains have been characterized. Truncation mutant
studies on the β2-Adrenergic receptor revealed a domain at the C-terminal end of the cytoplasmic tail that is involved in the recycling of receptors after agonist stimulated internalization and that this effect is abolished on deletion of that domain (63). The domain was identified as a Type I PDZ ligand that has the consensus sequence $\text{S/T-X-} \Phi$ ($\text{S}$-serine/$\text{T}$-threonine, $\text{X}$-any amino acid, $\Phi$-bulky hydrophobic residue). Apart from being involved in recycling of receptors these PDZ domain proteins have been shown to act as scaffolding proteins for signaling complexes and in mediating protein-protein interactions (62). A recent study on the Endothelin ET$_A$ receptor has also revealed the presence of internal PDZ binding domains that are different as compared to the C-terminal end PDZ binding domains (64). The proteins that bind to these PDZ ligands on GPCRs, apart from containing the PDZ domain also have additional functional domains that allow for multiple interactions.

Previous studies have shown that β-arrestins interact with phosphorylated residues on GPCRs and mediate both internalization and post-endocytic trafficking (19). The affinity of β-arrestin interaction delineates two classes of GPCRs, class A and B, which determines the pattern of resensitization of the receptors (Figure 5). Clusters of serine residues on Class B GPCRs have previously been shown to mediate stable interactions with β-arrestin in an agonist-dependent manner (51). Although, recently a study with the β2AR receptor showed that cluster of serines can also regulate transient interactions with β-arrestin (65). In addition to these commonly found motifs, other motifs like clusters of acidic residues, NPFX motifs and ubiquitin have been identified as sorting signals. Thus these various motifs present on GPCRs play an important role in their sorting and therefore ultimately their fate in a physiological context.
1.1.4 GPCRs as drug targets

Due to their highly complex and variable structure and their ubiquitous and tissue specific expression, GPCRs are ideal drug targets. More than 50% of drug targets are GPCR based today and generate worldwide sales exceeding 50 billion dollars (66). Use of agonists or antagonists to promote or inhibit the activity of GPCRs has been a common practice for drug therapy in most major organ systems (67). The most common GPCR targets are involved either in cardiovascular disease or in the central nervous system. Antagonists against the angiotensin II AT1 receptor (Losartan) are used for treatment of hypertension and heart failure. Antagonists against the adrenergic receptor family are mainly used to treat prostrate disorders, hypertension, and airway disease. Antagonists of the dopamine D3 receptor have been used to treat schizophrenia.

Deorphanizing of many GPCRs has led to the discovery of various drug targets and their therapeutic uses (68). As GPCRs have tissue specific expression, it is a challenge to target a drug only to a particular tissue and minimize side-effects. Such a problem can be circumvented by selectively distributing drugs through tissue specific metabolic pathways or drugs that act on a particular tissue type only. Another problem arises with the fact that many GPCRs regulate different pathways in different tissue types and hence use of common targeting drugs can have varying effects on different tissues. Bioinformatics and molecular modeling approaches are important in understanding receptor-ligand interactions and their affect on activity of GPCRs (69). The human angiotensin AT1 receptor antagonist can bind with high affinity to the rat AT1 receptor but not a frog AT1 receptor. Mutagenesis studies revealed that several amino acids critical to antagonist binding were missing. Gain of function experiments in frogs
restored binding abilities of the antagonist to the frog AT1 receptor (70). Such inter-species comparative approaches might also be useful in testing viability and development of new drugs.

Novel approaches towards designing new drug targets such as ligand-based modeling or combinatorial libraries need to be identified (71). Another novel method that has gained prominence in recent years is to base drug targets on the basal activity of GPCRs (72). This approach is relevant to this thesis project as in the second part, basal activity of the LPA₁ receptor has been investigated in detail. Basal activity for many adrenergic and opioid GPCRs have been reported and in particular μ-opioid receptor (MOR) basal activity has been hypothesized as being responsible for narcotic tolerance and dependence (73, 74). The use of inverse agonist that inhibits basal activity are of utmost importance in generating new drug strategies for GPCRs (75).

1.2 Lysophosphatidic Acid (LPA) and LPA Receptors

1.2.1 LPA: A bioactive molecule

Lysophosphatidic acid (LPA) (1-acyl-2-sn-glycerol-3-phosphate) is a naturally occurring, simple phospholipid abundant in serum (76). LPA had long been known as an intermediate in the lipid biosynthetic pathway and only in the past decade has been shown to be a bioactive, growth factor-like phospholipid (77). It is known to mediate a variety of cellular responses like cell proliferation (77), cell survival (78), aggregation of platelets (79), wound healing, smooth muscle contraction (80), cell invasion (81) and cytoskeletal reorganization (82). Although LPA seems to have growth factor like effects, it can also cause necrosis and apoptosis in some cases (83, 84). LPA has also been
implicated in embryonic development of vertebrae, cardiovascular and nervous systems (85, 86). As in the case of normal processes LPA has also been implicated in many abnormal processes such as cancer, atherosclerosis, airway disease and obesity (87-90), which will be discussed in the next section.

LPA is found in serum bound to albumin and gelsolin in physiologically relevant concentrations (91, 92). LPA is also found in other bodily fluids like saliva, follicular fluid, malignant effusions and mildly-oxidized LDL (90, 93). High concentrations of LPA (up to 25µM) are found in serum. LPA molecular species found in serum are mostly palmitoyl or oleoyl-LPA (94-96) of which the oleoyl species of LPA is the most potent activator of LPA receptors (97). Previous studies have also found that unsaturated LPA to be more potent than the saturated form such that the unsaturated form can induce smooth muscle cell proliferation and differentiation whereas the saturated form cannot (98, 99).

Although LPA can be generated through biochemical pathways in the cell, most of the LPA generated by platelets or adipocytes in serum is through the action of various enzymes such as lipases or kinases that cleave relatively complex phospholipids or phosphorylate monoacylglycerol (MAG) to give rise to LPA (Figure 6).

LPA can be generated by the action of phospholipases A1 (PLA1) and A2 (PLA2) by cleavage of a single fatty acyl chain from phosphatidic acid (PA). PLA1 removes the acyl chain from the sn-1 position generating unsaturated forms of LPA, whereas PLA2 cleaves the acyl chain in the sn-2 position generating saturated forms of LPA (100, 101). Although PLA2 can exist intracellularly (iPLA2) or extracellularly (sPLA2), sPLA2 cannot act on phosphatidic acid as PA is mostly found in membranes. Only upon cell membrane disruption can sPLA2 act upon PA to generate LPA.
Figure 5. Structure of LPA. Lysophosphatidic acid (LPA) has a single fatty acyl chain attached to the glycerol backbone at either the sn-1 or sn-2 positions. A phosphate group is attached at the sn-3 position. LPA can be generated by the action of various enzymes like phospholipases PLA1 and PLA2 and lysophospholipases like ATX/Lyso-PLD.
Therefore the discovery of yet another enzyme that could generate LPA from lysophospholipids was important in understanding LPA production in the external milieu.

LPA was also found to be generated from lysophosphatidylcholine (LPC) by the action of an enzyme called Lysophospholipase D (LysoPLD). This enzyme was later identified as the tumor promoting factor Autotaxin (102, 103). LysoPLD cleaves the choline head group from LPC and gives rise to LPA (Figure 6).

Autotaxin (ATX) was originally identified as an autocrine motility factor and is a transmembrane protein that is proteolytically cleaved to give rise to a soluble form of ATX/LysoPLD. The soluble form of ATX/lysoPLD can give rise to LPA locally and promote LPA-dependent tumor migration as the metastatic capability of breast cancer correlates with ATX/LysoPLD levels (104). Finally, a recently discovered mitochondrial enzyme acyl glycerol kinase (AGK) has been shown to generate LPA by phosphorylating MAG (105). Total LPA levels are also controlled by its metabolism. LPA can be broken down by various enzymes into different products. LPA can be dephosphorylated by lipid phosphate phosphatases (LPPs) to monoacylglycerol (MAG). LPPs are a major source of LPA “inactivators” and have been implicated in tumor amelioration, as expression of LPPs in ovarian cancer cells decreases colony formation and tumor growth (106). LPA can be converted to PA by LPA acyltransferases (LPAAT) by adding an acyl chain to either the sn-1 or sn-2 position. LPA generation and metabolism are not the only ways of modulating the effects of LPA. LPA, although water soluble, is also hydrophobic and hence can interact with other lipids and proteins in fluids or within cells. As mentioned
Figure 6. Biochemical pathways of LPA synthesis and degradation. PA, phosphatidic acid, LPX, lysophospholipid representing LPE, LPS or LPC, MAG, monoacylglycerol, PG, phosphatidylglycerol, PA–PLA1&2, PA-specific PLA1&2, Lyso-PLD, lysophospholipase D, LPP, lipid phosphate phosphatase, LPAAT, LPA acyltransferase, LPA–LPL, LPA-lysophospholipase, GPAT, glycerophosphate acyltransferase.
before, LPA can bind to serum albumin and gelsolin in nanomolar concentrations. Interestingly, albumin is used in most experiments as a carrier for LPA. Gelsolin can also bind to actin filaments and hence during local injury when platelets release LPA, gelsolin at the same time binds to actin filaments from damaged cells and therefore allows LPA to exert its effects (107). The liver fatty acid binding protein has also been identified as an intracellular carrier of LPA (108).

Therefore, knowledge of the processes involved in the metabolism of LPA itself can help in generating several therapeutic targets to inhibit the effects of LPA. As mentioned before, ATX, an LPA generating enzyme is a major tumor cell motility factor and hence is a candidate for pharmacological therapy. Additionally, LPP’s have been shown to inhibit ovarian cancer growth and hence is a target for pharmacological therapy in terms of increasing the activity of LPP’s.

### 1.2.2 LPA Receptors

Although LPA has many bioactive effects, there was considerable evidence that LPA mediated its effects through a G-protein coupled receptor pathway (109). The first LPA receptor gene was identified in the ventricular zone in the cerebral cortex, termed as ventricular zone gene-1 (vzg-1) (110). Based on amino acid similarity and ligand specificity two other receptor genes belonging to this family were identified and termed endothelial differentiation gene 2 and 7 (EDG-4 and EDG-7), which also included the receptors for sphingosine-1-phosphate, another lysophospholipid (111, 112). The three originally identified receptors were termed as LPA₁, LPA₂ and LPA₃ (EDG-2, EDG-4 and EDG-7). Interestingly, a mutant LPA₂ receptor was discovered with a frameshift
mutation isolated from an ovarian tumor cell line (113). This frameshift mutation (G deletion) results in a mutant LPA2 receptor that has four replaced and 31 additional amino acids compared to the normal LPA2 receptor. It is known that C-terminal tails of GPCRs are critical for a variety of interactions that regulate multiple processes and hence extra amino acids in the LPA2 tail might lead to abnormal interactions and abnormal phenotypes (114). Additionally, several variants of the LPA2 gene are observed in various cancer cell lines (111). Recently, a fourth human LPA receptor, LPA4/GPR23/P2Y9, was cloned (115). The LPA4 receptor has about 24% amino acid similarity to other LPA receptor family members and is evolutionary distant from them. LPA4 is more related to the nucleotide receptor P2Y family and interestingly the enzyme autotoxin generates ligands for both receptor families (116). Additionally, a fifth LPA receptor, LPA5/GPR92, was recently cloned, with a ~35% homology to LPA4 (117). These receptors have varying expression patterns in different tissues. The LPA1 receptor is the most widely expressed LPA receptor and is expressed in adult human organs like brain, heart, colon, small intestine, placenta, prostate, ovary, pancreas, testis and spleen, and lower expression levels in skeletal muscle and kidney (111, 116). Interestingly, LPA1 receptor expression was completely absent from the liver. Consequently, many LPA1 null cells derived from the liver, like RH7777 rat hepatoma cells and HepG2 cells have been used in elucidating receptor signaling pathways in response to LPA (118). The LPA1 expression pattern is very similar in both humans and mice (116). The LPA1 receptor is also differentially expressed in the brain both during development of the mouse embryo and after birth (119, 120). Unlike the LPA1 receptor, the LPA2 and LPA3 receptors have a more restricted expression pattern. LPA2 has abundant expression in testis and leukocytes
in humans and very low expression in pancreas, thymus, spleen and prostrate and no expression in adult liver, brain, heart, lung, kidney and ovary (111, 113, 120). Human LPA3 expression is strong in heart, prostrate, pancreas and testis and moderate in lung and ovary (112, 118). Human LPA4 is apparently weakly expressed in most tissues, except the ovary (115). Just like LPA4, LPA5 too has very low levels of expression in most tissues including brain and small intestine (117).

The LPA receptors being GPCRs, signal through heterotrimeric G-proteins and have been shown to activate different G-proteins (88, 116). LPA1 and LPA2 have been shown to activate Goi, Gaq and Gα12/13, whereas LPA3 can activate only Goi and Gaq but not Gα12/13 (88, 116). Activation of Goi and Gβγ and subsequently the MAPK/ERK pathway leads to cell proliferation and activation of the Pi3K-AKT pathway, promoting cell survival. Gaq activation leads to phospholipase C activation, which in turn causes Ca++ mobilization and PKC activation. Lastly, Gα12/13 mediates LPA-dependent Rho-GTPase activation and subsequent changes in the actin cytoskeleton (Figure 8). Additionally, the LPA1 receptor has been shown to activate Rac-GTPase through a Gβγ-PI3K pathway, which requires a Rac-GEF, TIAM1 (121, 122). In contrast, LPA4 and LPA5, which have more resemblance to nucleotide P2Y receptors, activate Gαs and adenylyl cyclase (115, 117). The LPA1 and LPA2 receptors have a PDZ-binding domain at their C-terminal ends, which have been shown to be required for interaction with PDZ-RhoGEF and Leukemia associated Rho GEF (LARG), both GEFs for Rho-GTPase (123). Both PDZ Rho-GEF and LARG apart from being GEFs for Rho also have a PDZ domain through which they interact with the LPA1 and LPA2 receptors.
Figure 7. G-protein signaling pathways activated by LPA. LPA signals through G-protein-coupled receptors via at least three distinct classes of heterotrimeric G proteins — Gq, Gi and G12/13 — leading to activation of multiple downstream effector pathways. Gq and/or Gi-mediated activation of phospholipase C (PLC), which leads to the hydrolysis of phosphatidyl inositol bisphosphate (PIP2), with consequent calcium mobilization and protein kinase C (PKC) activation; Gi-mediated activation of the RAS–ERK pathway, leading to cell proliferation; Gi-mediated activation of the PI3K–AKT (cell survival), which suppresses apoptosis; and G12/13-mediated activation of the RHO GTPases via specific exchange factors, RHOGEF, which leads to cytoskeletal remodeling (contraction and spreading), shape changes and cell migration.
Therefore, these PDZ-binding domains on LPA₁ and LPA₂ are required for Gα₁₂/₁₃ activation leading to Rho activation and actin cytoskeleton turnover. In contrast, LPA₃ lacks this PDZ-binding domain and does not activate Gα₁₂/₁₃.

Interestingly LPA₁ has been implicated in inducing cell motility of multiple cancer cell lines, which is mediated by the tumor cell motility-stimulating factor autotaxin/lyso-PLD by generation of LPA in the local environment (124). This correlates with the fact that LPA₁ has the ability to activate both Rac and Rho-GTPase leading to actin cytoskeleton turnover and regulate of cell migration. LPA₂, in addition, has also been shown to interact with PDZ domain proteins like MAGI-3, which regulate its ability to activate both ERK and RhoA (125). Additionally, monocytes and macrophages express both LPA₁ and LPA₂ receptors (126) and both these receptors could regulate migration of these cells. Receptor knock-out studies in mice have revealed an important role for LPA receptors. The LPA₁ receptor null mice, although not completely lethal, show certain birth defects like decreased olfaction (127). Embryonic fibroblasts derived from LPA₁ null mice show decreased cell migration, rounding and proliferation, which is consistent with the hypothesis that cells expressing LPA₁ confer migratory potential to cells expressing them. Most of the abnormal phenotypes observed in the LPA₁ null mice were limited and not completely present but LPA₁ and LPA₂ double null mice had more severe phenotypes suggesting that LPA-dependent processes are redundant (128). A recent study showed that in LPA₃ null mice litter size was significantly reduced due to improper implantation and spacing leading to delayed embryonic development and
lethality (129). This study adds to the increasing repertoire of processes regulated by LPA receptors.

Although much is known about the expression patterns and signaling pathways of the LPA\textsubscript{1} receptor, the physiological context of these phenomenon are not completely understood. Insight into many of the processes regulated by LPA receptors was derived from studies using receptor agonists and antagonists. Most of these agents have not been used in therapeutic studies due to their limited potency. Structural studies have revealed the importance of a single amino acid, glutamine, in the ligand recognition of the LPA versus S1P receptors (130). In addition to G-protein mediated effects of LPA, a recent study showed that the nuclear receptor PPAR-\(\gamma\) can also be activated by LPA (131). Another field that has not been extensively studied is LPA\textsubscript{1} receptor trafficking. Very few studies have elucidated the trafficking patterns of LPA\textsubscript{1} receptors, namely Murph et al., 2003 and Urs et al., 2005. A recent study also showed that LPA receptors can form both homo- and hetero-dimers, which could have an impact on the signaling patterns in response to LPA (132). Further investigations related to trafficking of LPA\textsubscript{1} receptors is described in this thesis dissertation.

1.3 Clinical significance of LPA and its cognate receptors

1.3.1 Cancer and Metastasis

The six alterations to cell physiology that represent the hallmarks of cancer are self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, unlimited growth potential, sustained angiogenesis and tissue invasion and metastasis (133). Initial studies with LPA clearly showed that it could induce cell motility
and invasiveness of various cell lines (81, 134, 135). A study with ovarian cancer patients revealed that the accumulated ascites fluid in those patients was enriched in a growth factor that stimulated growth of ovarian cancer cells and this factor was later on identified as LPA (136-138). After the discovery of cognate LPA GPCRs studies revealed that ovarian cancer cells had elevated expression of LPA2 and LPA3 receptors but not LPA1 receptors (138). In fact LPA1 was found to have an inhibitory effect on the growth of the ovarian cancer cells as its over-expression caused apoptosis of those cells (139). LPA can also induce anchorage-dependent and anchorage-independent growth of ovarian cancer cells as well as proliferation by activating the MAPK activity (140). Expectedly, LPA generating enzymes like ATX are up-regulated and LPA metabolizing enzymes like LPPs are down-regulated in ovarian cancer cells (141). LPA and other lysolipids have been shown to increase IL-8 expression in ovarian cancer cells affecting angiogenesis (142). Thus multiple signals at the same time can together alter cell physiology causing malignant transformation.

LPA has also been implicated in colon cancer. LPA2 receptors mediate mitogenic signals in human colon cancer cells whereas LPA1 enhances the metastatic potential of colon cancer cells (143, 144). Additionally, LPA mediates colon cancer proliferation through the β-catenin pathway via the LPA2 and LPA3 receptor (145). LPA has also been implicated in the survival, proliferation and migration of prostate cancer cells wherein the effects are mediated through the LPA1 receptor (146). LPA generating enzymes, like acyl glycerol kinase (AGK), in prostate cancer cells can also augment these effects through both epidermal growth factor receptor (EGFR) trans-activation and GPCR mediated activation. Expression of AGK in PC-3 prostate cancer cells enhanced cell proliferation
and migration in response to LPA (105). Additionally, the receptors that are involved in prostate tumor progression activate and interact with different proteins, thus increasing the complexity of these signaling pathways (147). Similarly, in breast cancer also LPA$_1$ expression is critical for mediating metastatic potential to cells (148). There is evidence suggesting that LPA supports the progression of ovarian and breast cancer metastasis to the bone (149). Additionally, this effect is mediated through the LPA$_1$ receptor as silencing of the receptor reduced bone destruction and metastasis (150). Interestingly, a study revealed that phorbol 12-myristate 13-acetate (PMA) could induce the production of LPA in ovarian and cervical cancer cells but not in breast cancer cells (151). In this thesis, studies done on the effects of PMA on LPA$_1$ receptor trafficking will be described and discussed.

1.3.2 Cardiovascular disease

Atherosclerosis is a disease state wherein any type of injury/insult or LDL particles causes lesions in the endothelium layer, which attract platelets and monocyte-derived macrophages into the intima (152). These macrophages can take up the oxidized LDL and swell up forming foam cells that occupy up to 6 layers of the intima of the artery. Rupture of these foam cells can then release lipids and oxidized LDL stored in the foam cells causing more macrophages and platelets to accumulate at the neo-intima. This results in the formation of an atherosclerotic plaque that blocks the artery and subsequently blocks flow of blood causing myocardial infarction (152). LPA has been implicated in cardiovascular disease especially in formation of atherosclerotic plaques (153). LPA was found to be enriched in mildly-oxidized LDL (mox-LDL) and that it
activated platelets and endothelial cells in the surrounding environment (90). Additionally, LPA was also found to be enriched in the lipid core of the neo-intima and that it caused the accumulation of platelets and macrophages (90, 154). It was hypothesized that upon rupture of the neo-intima, the release of mox LDL and LPA could result in accumulation of additional platelets and macrophages in the lesion leading to blockage of the artery. Previous studies have shown that monocyte-derived macrophages express both the LPA\textsubscript{1} and LPA\textsubscript{2} receptors (155) and it is possible that the presence of LPA could act as a chemo-attractant to these macrophages. Additionally, the LPA species found in the neo-intima are predominantly of the acyl form (18:0) and could have varying platelet activating potential (156). Interestingly, effects of LPA present in the neo-intima, like platelet shape change and aggregation of platelets and macrophages, were blocked by LPA receptor antagonists (156). Although LPA GPCRs might have a role in the formation of the atherosclerotic plaque, increasing evidence points towards PPAR-γ as a more important player in the formation of a neo-intima (153). Previous studies had shown that the major LPA species found in the neo-intima were the acyl form (18:0) and that its activation potential is variable. However, recent studies revealed that the unsaturated alkyl forms but not the acyl forms of LPA promoted neo-intima formation. It was proposed that this effect was not through the LPA GPCRs but instead was through activated PPAR-γ and that it was responsible for vascular smooth muscle (VSMC) differentiation and neo-intima formation (98, 99, 157). Therefore LPA might have a dual role in the formation of neo-intima through PPAR-γ and atherosclerotic plaque formation through the EDG/LPA receptors. Inhibitors of the LPA GPCRs had a modest effect on neo-intima formation but inhibitors of PPAR-γ had a more robust inhibitory effect on
neo-intima formation (157). Thus further studies, both with LPA GPCR and PPAR-γ, need to be done to gain insight into this dual role for LPA in atherosclerosis.

1.3.3 LPA in the nervous system

Initial studies in the field of LPA were done in the nervous system leading to the discovery of the first LPA receptor (LPA₁/VZG-1/EDG-2). These studies showed that LPA could induce neurite retraction (158). The presence of LPA receptors in the nervous system is evidence enough that LPA might play an important role. LPA₁ and LPA₂ are the major receptors expressed in the nervous system. Studies with LPA₁ and LPA₂ null mice have revealed a requirement for LPA signaling as these null mice exhibit abnormal phenotypes like cranial deformities and impaired suckling behavior (127, 159). LPA₁ null mice also seem to have decreased serotonin levels, although there were no behavioral changes observed (128). A recent study revealed a new role for LPA signaling in neuropathic pain (160). Injury or treatment with LPA caused hyperalgesia and allodynia through the Rho pathway and these effects were absent in the LPA₁ null mice (159). Additionally, LPA has been implicated in the myelination of nerves. LPA₁ receptors have been found in mature oligodendrocytes as well as schwann cells (161, 162). A recent study has also indicated a role for LPA signaling in psychiatric disorders like schizophrenia (163). The time-dependent expression of LPA receptors in the brain indicates a developmental role for LPA signaling. LPA causes thickening and abnormal folding of the cortical wall, which could be attributed to LPA-induced mitosis and lack of apoptosis (164). Although LPA receptor knockout studies have revealed a role for LPA signaling in the nervous system, most of the phenotypes are not lethal if the mice survive
and hence indicate redundancy in signaling, which is compensated by some other pathways (165). It is possible that a combination of lysophospholipid pathways including that of the S1P receptors along with the LPA receptors regulate the different functions in the nervous system.

1.3.4 LPA and Obesity

Obesity is defined as an energy storage disorder where energy imbalance causes weight gain, with excess calories stored as triglycerides in adipose tissue (166). Prevalence of obesity also has a strong correlation with type 2 diabetes and cardiovascular disease (167). Adipocytes are the major cell type involved in obesity and have been shown to be endocrine cells that release various cytokines as well as autocrine and paracrine factors (168). Adipocytes have been shown to generate LPA through the secretion and action of ATX/Lyso-PLD (89, 169). These studies demonstrated that LPA induced pre-adipocyte differentiation and cell proliferation and additionally, genetically obese diabetic mice seemed to overexpress ATX in them. In normal weighing adults the ratio of pre-adipocytes to adipocytes is a tightly regulated process that might be disrupted due to excessive LPA signaling. Surprisingly, a recent study also showed that the LPA induced down-regulation of PPAR-γ2 expression through the action of the LPA₁ receptor and reduced triglyceride accumulation (170). This study also showed that although LPA₁ null mice were leaner than wild-type mice, they had more pre-adipocyte content. ATX expression is highly up-regulated in newly differentiated adipocytes with a concurrent increase in the release of LPA. Thus, taken together, these studies indicate that LPA has an anti-adipogenic activity on pre-adipocytes but has a proliferative and differentiating
effect on pre-adipocytes when generated by adipocytes through ATX/Lyso-PLD activity. Other factors such as Insulin and IGF-1 also contribute to the differentiation of pre-adipocytes to adipocytes. Therefore, further studies might provide a link between type-2 diabetes and obesity and the role of LPA signaling.

In the following chapters the studies on the trafficking of the LPA1 receptor will be described and discussed.
PART I:

A REQUIREMENT FOR MEMBRANE CHOLESTEROL IN THE β-ARRESTIN- AND CLATHRIN-DEPENDENT ENDOCYTOSIS OF LPA₁ LYSOPHOSPHATIDIC ACID RECEPTORS
CHAPTER 2
INTRODUCTION

Lysophosphatidic acid (LPA, 1-acyl-2-lyso-sn-glycero-3-phosphate) is an abundant serum mitogen that evokes growth factor-like responses in many cell types through activation of G-protein-coupled receptors (GPCR) (86). LPA signaling affects a variety of cellular functions including: growth stimulation (cell proliferation and cell survival) (171-173), induction of cytoskeletal rearrangements via Rho GTPases (174), stimulation of serum-responsive genes (175), neurite retraction (158), promotion of tumor cell migration/invasion (176) and the secretion of peptide growth factors (142, 177, 178).

Most of the effects of LPA are mediated through the activation of three members of the endothelial differentiation gene superfamily of receptors: LPA1, LPA2 and LPA3 (86, 179). Upon LPA binding, both LPA1 and LPA2 activate the Gi, Gq and G12/13 families of heterotrimeric G proteins; LPA3 only activates Gi and Gq (180). In addition to these well characterized GPCRs, LPA also stimulates the orphan receptor, GPR23/LPA4 (115) and the non-GPCR target, peroxisome proliferator-activated receptor γ (131). Given the complexity of cellular responses to LPA signaling and the potential role of LPA receptor subtypes in various cancers (88), it is important to understand the mechanisms that regulate the activity of individual LPA receptors.

Upon agonist stimulation, most GPCRs are rapidly internalized into cells through a variety of different endocytic pathways. This facilitates either receptor down-regulation or receptor resensitization (13). Agonist stimulation usually leads to the rapid phosphorylation of serine/threonine residues located within cytoplasmically exposed
regions of GPCRs (1). This subsequently induces the binding of β-arrestin proteins, which results in signal attenuation and often targets the GPCR to clathrin-coated pits for endocytosis (181). Internalized GPCRs transit through the endosomal system and are either sorted to lysosomes for degradation or become dephosphorylated by membrane-associated phosphatases and are recycled back to the plasma membrane (1). In addition to clathrin-mediated endocytosis, many GPCRs utilize a variety of clathrin-independent internalization mechanisms including cholesterol-dependent pathways such as caveolae (25). Also, β-arrestins are not universally required for GPCR endocytosis as shown for the thrombin receptor, PAR1, whose association with β-arrestins is required for signal attenuation but not for its endocytosis (182). Thus, the mechanisms that regulate both signal attenuation and receptor endocytosis can vary from one GPCR to another.

We have previously shown that LPA₁ is probably internalized by clathrin-dependent endocytosis as dominant negative mutants of dynamin 2 (K44A) and Rab 5 (S34N), which regulate clathrin-dependent trafficking, strongly inhibited LPA₁ endocytosis (183). However, a recent study showed that LPA stimulation of the phosphoinositide 3-kinase (PI3-K)/Akt pathway was dependent upon membrane cholesterol (184) suggesting a positive role for cholesterol-rich plasma membrane microdomains in LPA signaling. As cholesterol enriched microdomains, such as caveolae, can also mediate receptor endocytosis, it is not clear what the relationship is between LPA signaling from cholesterol-rich microdomains and the endocytosis of LPA receptors. To address this question and to gain a better understanding about the regulation of LPA receptors, we investigated the role of membrane cholesterol, β-arrestins and clathrin in the signaling and endocytosis of the ubiquitously-expressed LPA₁ receptor.
CHAPTER 3
MATERIALS AND METHODS

3.1 Antibodies and reagents
Lysophosphatidic acid (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate; LPA) was purchased from Avanti Polar Lipids (Alabaster, AL). Isoproterenol and cytochalasin D was obtained from Sigma Chemical Co. (St Louis, MO) and carbachol from Fluka Chemika-Biochemika. FLAG-tagged LPA$_1$ receptors were detected with mouse anti-FLAG antibodies (Sigma, St Louis, MO); HA-tagged β2AR and HA-tagged M1 muscarinic acetylcholine receptor (mAChR) were detected with mouse anti-HA antibodies (Covance, Berkeley, CA). Alexa 488- labeled transferrin (Alexa 488-Tfn), Alexa 594- and Alexa 488- conjugated goat anti-mouse were purchased from Molecular Probes (Eugene, OR). Monoclonal antibodies to clathrin heavy chain and monoclonal anti-actin antibodies were purchased from BD Transduction labs (San Jose, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Mouse anti-AP2 antibodies were purchased from Affinity Bioreagents (Golden, CO). FITC-labeled anti-CD59 was obtained from Chemicon (Temecula, CA). Methyl-β- cyclodextrin and water-soluble cholesterol complexes were purchased from Sigma. myo-[3H]inositol was purchased from American Radiolabeled Chemicals (St Louis, MO).

3.2 Cell culture and DNA transfection
HeLa cells stably expressing the LPA$_1$ receptor (termed LPA$_1$/HeLa cells), native HeLa cells, wild-type (WT) mouse embryo fibroblasts (MEFs) and β-arrestin 1/2 KO MEF
cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin (Media Tech, Herndon, VA) and 1 mM sodium pyruvate (Biosource International, Camarillo, CA) at 37°C with 5% CO2. Cells were grown on glass coverslips (for immunolocalization) and transfected in six-well dishes, or were grown in 24-well dishes (for myo-[3H]inositol labeling) using Lipofectin or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer’s directions. Plasmids encoding HA-tagged β2AR, β- arrestin 1-GFP, β-arrestin-2-GFP, HA-tagged M1 mAchR were transiently transfected at 1.0 µg/well (in six-well plates) and have been previously described (Paing et al., 2002; Scott et al., 2002).

3.3 siRNA-mediated reduction of clathrin

siRNA oligonucleotides to clathrin were purchased from Dharmaco (Lafayette, CO) and have been described previously (185). LPA1/HeLa cells were transiently transfected with 300 pmol (10 cm dish) or 100 pmol (24-well plate) of siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection medium was replaced with complete medium (without penicillin/streptomycin) 5 hours later and the cells were incubated for 16 hours. The cells were transfected a second time as above and the medium was then replaced with serum-free medium (SFM) and incubated for an additional 16 hours before experimentation.
3.4 Indirect immunofluorescence

Cells were treated as described in the figure legends, 24-48 hours after transfection. Cells were then fixed in 2% formaldehyde in phosphate buffered saline (PBS) for 10 minutes and rinsed with 10% fetal bovine serum (FBS) containing 0.02% azide in PBS (PBS-serum). Fixed cells were incubated with primary antibodies diluted in PBS-serum containing 0.2% saponin for 45 minutes and then washed (three times, 5 minutes each) with PBS-serum. The cells were then incubated in fluorescently labeled secondary antibodies diluted in PBS-serum containing 0.2% saponin for 45 minutes, washed three times with PBS-serum, washed once with PBS and mounted on glass slides as previously described (186). For Alexa 594-Tfn and FITC-labeled anti-CD59 internalization, LPA1/HeLa cells were briefly rinsed three times with 0.5% bovine serum albumin (BSA) in SFM and incubated in the same medium for 30 minutes at 37°C. The cells were then incubated with Alexa 594- conjugated human transferrin (50 µg/ml) or FITC-conjugated anti-CD59 (1 µg/ml) for 30 minutes at 37°C in the presence or absence of 10 µM LPA. Antibodies bound to the cell surface were removed by rinsing the cells with 0.5% acetic acid, 0.5 M NaCl, pH 3.0 solution (for Alexa 488-Tfn) or 100 mM glycine, 20 mM magnesium acetate, 50 mM KCl, pH 2.2 (for FITC anti-CD59) (187). Cells were rinsed in complete medium, fixed and processed for fluorescence microscopy. For assessing uptake in the presence of methyl-β-cyclodextrin (5 mM) or nystatin (50 µg/ml), cells were pretreated with DMEM supplemented with 0.5% BSA, with or without drugs, for 60 minutes prior to antibody and/or LPA addition. All images were acquired using an Olympus BX40 epifluorescence microscope equipped with a 60X Planapo lens and photomicrographs were prepared using an Olympus MagnaFire SP digital camera.
(Olympus America, Melville, NY). Images were processed with Adobe Photoshop 6.0 software.

3.5 Quantification of LPA₁ colocalization with internalized Alexa- Tfn
Stably-transfected LPA₁/HeLa cells or transiently-transfected HeLa cells expressing M₁ mAChRs were grown on glass coverslips and treated with MβCD and/or water-soluble cholesterol as described in the figure legends. The cells were then incubated with 50 µg/ml Alexa 594-Tfn for 30 minutes in the presence or absence of 10 µM LPA or 1 mM carbachol, respectively. The cells were rinsed with a mild acid wash as described above, fixed with 2% formaldehyde in PBS and processed for immunofluorescence localization of LPA₁ using M₁ mouse anti-FLAG IgG or M₁ mAChR using mouse anti-HA IgG followed by Cy2 secondary antibodies. The extent of LPA₁ or M₁ co-localization with internalized Alexa 594-Tfn was determined by quantifying the extent of pixel co-localization of GPCR staining with Alexa 594-Tfn fluorescence using Metamorph Imaging software (Universal Imaging, West Chester, PA) as described (186, 188). The background was subtracted from unprocessed images and the percentage of GPCR pixels that overlapped with Alexa-Tfn pixels was measured. The data is presented as the mean±s.e.m. of measurements from 20 cells per sample from a representative experiment that was performed three independent times with similar results.

3.6 Immunoblotting
Following 72 hours of siRNA treatment, cells were solubilized by addition of lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium
phosphate pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 M sodium orthovanadate, 0.02% azide, 100 µg/ml leupeptin and 0.1 mM PMSF) and incubated on ice for 60 minutes. The samples (12 µg protein per lane) were then separated by 10% SDS-PAGE and transferred to nitrocellulose. Clathrin heavy chain was detected using mouse anti-clathrin antibodies and actin was detected using monoclonal anti-actin antibodies. The binding of primary antibodies was detected by using an enhanced chemiluminiscence detection kit (Amersham Biosciences, Piscataway, NJ).

3.7 Phosphoinositide hydrolysis

LPA₁/HeLa cells or mouse embryo fibroblasts derived from wild-type or β-arrestin 1/2 null mice were plated at a density of 4.0X10⁴ cells/well into 24-well plates and transfected with plasmids encoding wild-type LPA₁ or M1 mAChRs alone or in combination with plasmids encoding wild-type β-arrestin 2 using Lipofectamine 2000. Transient transfection of plasmids encoding M1 mAChRs was performed by using Lipofectin reagent. At 24 hours post-transfection, cells were labeled overnight with myo-[³H]inositol in inositol- and serum-free medium, treated as described in the figure legends and then processed for analysis of phosphoinositide hydrolysis by anion exchange chromatography as described (182).

3.8 Triton X-100 extraction of cells

LPA₁/HeLa cells were plated onto glass coverslips in 35 mm dishes at a density of 0.2x10⁶ cells per plate. After allowing cells to attach for 24 hours, the medium was changed to serum-free medium and the cells were incubated overnight (~16 hours). The
following day, the cells were treated as described in the figure legends and subsequently incubated with ice-cold 1% Triton X-100 in PBS on ice for 3 minutes prior to fixation with ice-cold 2% formaldehyde in PBS. LPA$_1$ or was localized using indirect immunofluorescence microscopy. To monitor the fate of surface LPA$_1$, LPA$_1$/HeLa cells were incubated on ice with mouse anti-FLAG antibodies for 30 minutes after LPA treatment to label only surface LPA$_1$. These cells were then extracted with 1% Triton X-100, fixed and processed for immunofluorescence localization as described above. Relative receptor expression was quantified by measuring receptor pixel intensity using MetaMorph imaging software and was normalized to DNA content, labeled with Hoescht dye.

3.9 Whole-cell ELISA quantification of surface LPA$_1$

LPA$_1$/HeLa cells were plated in 24-well dishes (Falcon) at a density of 0.4x10$^5$ cells per well and grown overnight. Cells were then transiently transfected with no siRNA or with 100 pmol/well of clathrin-specific siRNA (185) using Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). After 24 hours, the cells were again transfected with 100 pmol/well of clathrin-specific siRNA or no siRNA. 24 hours later, the cells were incubated in the presence or absence of 10 µM LPA for 45 minutes and fixed in 2% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes and rinsed with 10% fetal bovine serum (FBS), containing 0.02% azide, in PBS (PBS-serum). Fixed cells were incubated with mouse anti-M1 FLAG primary antibody diluted in PBS-serum (250 µl/well) for 1 hour and then washed (three times, 5 minutes each) with PBS-serum. The cells were then incubated with horseradish peroxidase-
conjugated goat anti-mouse IgG secondary antibody (Pierce Biotechnology, Rockford, IL) diluted in PBS-serum (250 µl/well) for 1 hour, washed three times with PBS-serum and washed three times with PBS. The cells were then incubated for 1 hour at 37°C with ABTS (2,2 β-Azinobis [3-ethylbenzothiazoline-6- sulfonic acid]-diammonium salt) (Pierce Biotechnology). A 200 µl aliquot was then removed from each well, transferred to a 96-well plate and the absorbance read at 405 nm (corrected for blank). Internalization is expressed as the percent difference in surface LPA1 between unstimulated cells and agonist-stimulated cells. The data are the mean±s.e.m. of six replicates/siRNA sample combined from two independent experiments.

3.10 Cholesterol measurements

HeLa cells, stably expressing FLAG-tagged LPA1, were seeded in sixwell plates at a density of 0.5×10^6 cells per well, allowed to attach overnight and then incubated with serum-free DMEM for 24 hours prior to treatment. The cells were treated for 60 minutes, as described in the figure legend, rinsed twice with ice-cold PBS (pH 7.4) and then solubilized in ice-cold PBS (pH 7.4) containing 1% Triton X-100 and protease inhibitor cocktail (2 mM AEBSF, 1 mM EDTA, 130 µM bestatin, 14 µM E-64, 1 µM leupeptin and 0.3 µM aprotinin). Total cellular cholesterol was quantified using an Amplex Red Cholesterol Assay Kit (Molecular Probes; Eugene, OR), as indicated by the manufacturer. Briefly, cholesterol esters in the cell extracts are hydrolyzed by cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase to yield H2O2, which is detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red). In the presence of horseradish peroxidase (HRP), Amplex Red reacts with H2O2 to produce
fluorescent resorufin. Fluorescence was measured with a fluorescence microplate reader using excitation at 560 nm and fluorescence detection at 590 nm and total cholesterol was calculated from a standard curve using purified cholesterol. Cellular cholesterol was normalized to total protein concentration, which was quantified by BCA Protein Assay (Pierce Biotechnology).

3.11 Statistical analysis

The data is expressed as the mean±s.e.m. from the indicated number of independent experiments performed in triplicate. Differences were analyzed by two-factor ANOVA followed by a Tukey’s statistical significance test.
CHAPTER 4

RESULTS

4.1 LPA₁ is internalized by clathrin-mediated endocytosis

Previous work from our lab had suggested that LPA₁ was internalized by clathrin-dependent endocytosis based on the inhibitory effects of mutant dynamin 2 K44A and Rab 5 S34N (186). However dynamin K44A can also inhibit endocytosis from cholesterol-rich caveolae (186, 189, 190). To directly test whether LPA₁ used a clathrin-dependent pathway, we determined the effects of reducing the cellular abundance of the clathrin heavy chain, using small interfering RNAs (siRNAs), on the endocytosis of LPA₁. Figure 8A shows the distribution of FLAG-tagged LPA₁ in stably transfected HeLa cells (LPA₁/HeLa cells). In untreated cells, LPA₁ is localized predominantly at the plasma membrane and to a lesser extent, at the Golgi complex, which probably represents newly synthesized LPA₁ en route to the plasma membrane. Treatment with 10 µM LPA results in a redistribution of LPA₁ to numerous punctate structures, which we have previously shown to colocalize with transferrin receptor-positive endosomes (186). To investigate the role of clathrin in LPA₁ endocytosis, we adapted a double-transfection procedure that was previously described (185) to knock down clathrin heavy chain amounts in HeLa cells to near undetectable levels. Using this procedure, we observed a 73% reduction in the abundance of clathrin relative to mock-transfected siControl cells (Figure 8B) in LPA₁/HeLa cells. Treatment of cells with clathrin siRNA did not alter the abundance of actin.
Figure 8. siRNA-mediated reduction of clathrin inhibits agonist-induced endocytosis of LPA₁. (A) Stably-transfected LPA₁/HeLa cells were incubated in the presence or absence of 10 µM LPA for 30 minutes, fixed and processed for immunofluorescence detection of FLAG-tagged LPA₁ with M1 mouse anti-FLAG antibodies and fluorescently-labeled secondary antibodies. (B) Cell lysates were prepared from stably transfected LPA₁/HeLa cells, which were either mock transfected (siControl) or transfected with clathrin siRNA (siClathrin) for 48 hours, separated by SDS-PAGE and immunoblotted for clathrin heavy chain (CHC) or actin. (C) Stably transfected LPA₁/HeLa cells grown in 24-well plates were either mock transfected (siControl) or transfected with clathrin siRNA (siClathrin) for 48 hours prior to treatment with or without 10 µM LPA for 45 minutes. The cells were fixed and processed for whole-cell ELISA to quantify surface LPA₁ receptors as described in Materials and Methods. LPA₁ internalization is expressed as the percentage difference in surface LPA₁ between unstimulated cells and agonist-stimulated cells. The data are the mean±s.e.m. of six replicates/siRNA sample combined from two independent experiments. **P<0.01 compared to levels in the siControl. (D) Stably transfected LPA₁/HeLa cells were treated with clathrin siRNA for 48 hours prior to incubation with FITC-labeled mouse anti-CD59 and Alexa 594-Tfn for 30 minutes and fluorescence visualization of anti-CD59 and Alexa 594-Tfn labeling. Bar, 10 µm.
Endocytosis of LPA₁ was quantified by using a whole-cell ELISA, which measures the agonist-induced loss of cell surface LPA₁ (182, 191). In siControl cells, 10 μM LPA induced LPA₁ internalization (~30%) and this was strongly inhibited in siClathrin cells (~2%) (Figure 8C). In siClathrin cells, both the agonist-stimulated internalization of β2-adrenergic receptors (β2ARs) (data not shown), which are known to use clathrin-dependent mechanisms (192) and the constitutive endocytosis of Alexa transferrin was strongly inhibited (Figure 8D). As a negative control, we examined the effects of clathrin knockdown on the endocytosis of anti-CD59 antibodies bound to endogenous CD59, which is internalized via cholesterol rich, detergent-resistant membranes and then merges with a clathrin-independent trafficking pathway that is regulated by the Arf6 GTPase as shown by Naslavsky et al. (187). LPA₁/HeLa cells were transfected either with or without clathrin siRNAs and then incubated with FITC-labeled mouse anti-CD59 antibodies along with Alexa 594-Tfn for 30 minutes. The cells were acid-stripped to remove surface-bound anti-CD59 antibodies and Alexa 594-Tfn. In cells transfected with clathrin siRNAs, FITC-labeled anti-CD59 antibodies localized to pleomorphic tubulovesicular structures (Figure 8D, Anti-CD59) similar to those described (187). As expected, these same siRNA-treated cells did not internalize Alexa 594-Tfn (Figure 8D, Alexa 594-Tfn). Taken together, these results indicate that LPA₁ is internalized by clathrin-mediated endocytosis.

4.2 β-arrestins are critical for LPA₁ signal attenuation and receptor endocytosis

Clathrin-mediated endocytosis of many GPCRs is also dependent upon their association with the multi-functional β-arrestins (13, 22). β-arrestin binding is initiated
through the agonist-induced phosphorylation of cytoplasmic serine/threonine residues in the GPCR by G protein receptor kinases (GRKs) such as GRK2 (7). β-arrestin binding promotes both receptor desensitization, by preventing receptor-G protein coupling and clathrin-dependent endocytosis of the receptor. To determine whether β-arrestins are required for LPA₁ endocytosis, we compared agonist-stimulated internalization of LPA₁ and β2AR in mouse embryo fibroblasts (MEFs) derived from either wild-type or β-arrestin 1 and 2 null mice (193) (Figure 9). Wild-type MEFs were transiently transfected with plasmids encoding either LPA₁ or β2ARs and then incubated in the presence or absence of agonist. In the absence of agonist treatment, both receptors were primarily localized to the plasma membrane in a diffuse pattern (Figure 9A, untreated). Upon agonist treatment for 30 minutes, both LPA₁ and β2ARs redistributed to small punctate endosomal structures dispersed throughout the cell. The labeling of these structures was not observed in non-permeabilized cells, thus indicating that they were internal endosomal structures (Figure 9A, non-permeabilized). In contrast to wild-type MEFs, agonist treatment of β-arrestin 1/2 KO MEFs expressing either LPA₁ or β2ARs did not lead to their endocytosis (Figure 9B, +agonist). Expression of wild-type β-arrestin-2-GFP in the knockout cells restored agonist-induced endocytosis of both LPA₁ (Figure 10, 30 minutes) and β2ARs (data not shown), thus indicating that β-arrestins were required for the endocytosis of LPA₁ as well as β2ARs. Previous studies have shown that agonist stimulation of different GPCRs leads to the translocation of cytosolic β-arrestin proteins to the plasma membrane (194).
Figure 9. Agonist-induced endocytosis of LPA$_1$ is inhibited in β-arrestin 1/2 double knockout mouse embryo fibroblasts. (A) Wild-type MEFs were transiently transfected with plasmids encoding either FLAG-tagged LPA$_1$ or HA-tagged β2AR and then incubated in the presence or absence of agonist (10 μM LPA or 20 μM isoproterenol, respectively) for 30 minutes prior to indirect immunofluorescence localization of the receptor proteins either in the presence or absence of detergent permeabilization. (B) β-arrestin ½ double knockout MEFs were transiently transfected with plasmids encoding either FLAG-tagged LPA$_1$ or HA-tagged β2ARs and incubated in the presence or absence of agonist, as above, prior to indirect immunofluorescence localization of the receptor proteins. Bar, 10 μm.
For some GPCRs such as β2ARs, this association with β-arrestins is transient and is not observed following receptor endocytosis, whereas other GPCRs, such as angiotensin AT1a receptors and vasopressin receptors, maintain a stable association with β-arrestins even on endosomes after endocytosis (19). To determine whether LPA1 formed a transient or stable association with β-arrestins, we examined the distribution of LPA1 and β-arrestin-2-GFP after 0, 2 and 30 minutes of LPA treatment (10 µM) (Figure 10). In untreated cells, LPA1 localized to the plasma membrane and β-arrestin-2-GFP localized in a diffuse cytoplasmic pattern (Figure 10, Untreated). After 2 minutes of LPA treatment, LPA1 localized to small punctate structures, which partially co-localized with β-arrestin-2-GFP (Figure 10, inset, arrows). However, many punctate structures contained LPA1 but did not contain β-arrestin-2-GFP, particularly in the larger and more pleotrophic structures. Following 30 minutes of LPA treatment, LPA1 localized to heterogeneously sized endosomal structures, but β-arrestin-2-GFP returned to the diffuse cytoplasmic pattern observed in untreated cells (Figure 10, 30 minutes). This suggested that β-arrestins dissociate from LPA1 receptors at or near the cell surface and do not form a stable association with β-arrestin proteins, as defined by Oakley et al. (19, 31). Taken together, these data indicate that β-arrestins are critical for the endocytosis of LPA1 and that LPA1 only transiently associates with β-arrestins at the cell surface. As mentioned above, β-arrestin binding to activated GPCRs leads to signal attenuation (195). We next investigated whether β-arrestins were important for the desensitization of LPA1.
Figure 10. Re-expression of wild-type β-arrestin 2 GFP in β-arrestin knockout MEFs restores LPA₁ receptor endocytosis. (A) β-arrestin 1/2 double knockout MEFs were transiently transfected with plasmids encoding LPA₁ and wild-type β-arrestin-2-GFP. Cells were then incubated with 10 μM LPA for 0, 2, or 30 minutes prior to fixation and indirect immunofluorescence. The inset shows a magnified image of the boxed region and the arrows indicate punctate structures that co-label for both LPA₁ and β-arrestin-2-GFP. Note that the recruitment of β-arrestin2-GFP to these punctate structures is transient, observable after 2 minutes of LPA treatment but not after 30 minutes of LPA treatment.
Figure 11. Re-expression of wild-type β-arrestin 2 GFP in β-arrestin knockout MEFs restores LPA₁ signal attenuation. MEFs derived from wild type (WT) or β-arrestin 1/2 null (βArr 1/2KO) mice were transfected with plasmid encoding wild-type LPA₁ receptors; β-arrestin 1/2 null MEFs were also co-transfected with plasmids encoding LPA₁ and wild-type β-arrestin 2 (βArr 1/2KO + LPA₁ + WT βArr2). Cells were then labeled with [3H]myo-inositol overnight in serum-free medium and incubated for 1 hour in the absence (Untreated) or presence of 10 μM LPA prior to analysis of phosphoinositide hydrolysis, as described in Materials and Methods. The radioactivity recovered in the different samples was normalized to total cellular protein and the data are presented as the mean±s.e.m. of triplicate measurements from a representative experiment that was repeated three times. **P<0.01, comparison of LPA-stimulated phosphoinositide hydrolysis in β-arrestin 1/2 KO MEFs to that observed in WT MEFs. Bar, 10 μm.
We examined the ability of LPA\textsubscript{1} receptors, which activate Gi, Gq and G12/13 signaling pathways (Fukushima et al., 1998), to promote phosphoinositide (PI) hydrolysis, via Gq stimulation of phospholipase C, in the wild type and \(\beta\)-arrestin 1/2 KO MEFs (Figure 11). LPA\textsubscript{1}-transfected wild type and \(\beta\)-arrestin 1/2 KO MEFs were labeled with [3H]inositol and the accumulation of inositol phosphates was determined in untreated cells and cells treated with 10 \(\mu\)M LPA for 60 minutes at 37\(^{\circ}\)C. LPA treatment increased the accumulation of [3H] inositol phosphates in wild-type MEFs by \(\sim\)2.5-fold. However, stimulation of LPA\textsubscript{1}-transfected \(\beta\)-arrestin 1/2 KO MEFs led to a 4.3-fold increase in inositol phosphate accumulation, suggesting that \(\beta\)-arrestins are important for attenuation of LPA signaling. To further test this, we determined the effects of re-expression of wild-type \(\beta\)-arrestin 2 on inositol phosphate accumulation in LPA\textsubscript{1}-transfected \(\beta\)-arrestin 1/2 KO MEFs (Figure 11). Co-transfection of wild-type \(\beta\)-arrestin 2 and LPA\textsubscript{1} in the \(\beta\)-arrestin 1/2 KO MEFs reduced the magnitude of LPA-induced inositol phosphate accumulation to 3.1-fold, which was similar to that observed in LPA\textsubscript{1}-transfected WT MEFs (2.5-fold). Taken together, these observations strongly support the notion that \(\beta\)-arrestin association with LPA\textsubscript{1} receptors is important for signal attenuation and for clathrin-mediated receptor internalization.

4.3 Membrane cholesterol is required for LPA\textsubscript{1} signaling and receptor endocytosis

Having established that the agonist-induced endocytosis of LPA\textsubscript{1} was mediated by a \(\beta\)-arrestin- and clathrin-dependent pathway, we next investigated the role of membrane cholesterol in LPA\textsubscript{1} signaling and trafficking. As mentioned, membrane cholesterol has been shown to be important for LPA stimulation of PI3-kinase/Akt signaling (184). To
address this question, we first examined the effects of cholesterol extraction with methyl-β-cyclodextrin (MβCD) and the effects of cholesterol disruption with the cholesterol binding drug, nystatin, on LPA stimulation of phosphoinositide hydrolysis, which is stimulated by Gq signaling. As a first step, we measured the effects of these cholesterol-perturbing drugs and the effects of water-soluble cholesterol:MβCD complexes on the cellular abundance of cholesterol in LPA₁/HeLa cells by using a quantitative cholesterol measurement assay (see Materials and Methods) (Table 1). Control LPA₁/HeLa cells contained 17.10±0.13 µg cholesterol/mg protein and treatment with 5 mM MβCD for 1 hour reduced cellular cholesterol by 62% to 6.5±0.05 µg cholesterol/mg protein. Addition of 10 mM cholesterol, as a water-soluble MβCD complex, for 1 hour after MβCD extraction, increased cellular cholesterol levels to approximately twice that observed in control cells (34.2±0.78 µg cholesterol/mg protein). In contrast, treatment of LPA₁/HeLa cells with 50 µg/ml nystatin for 1 hour slightly elevated the amount of cellular cholesterol (20.8±1.5 µg cholesterol/mg protein) and addition of water-soluble cholesterol to nystatin-treated cells increased cellular cholesterol amounts by approximately 2.5-fold relative to control LPA₁/HeLa cells (42.9±1.3 µg cholesterol/mg protein). This is consistent with the notion that nystatin merely binds sterols but does not extract them from cells. We next examined the effects of these cholesterol-perturbing drugs on LPA₁ stimulation of phosphoinositide hydrolysis, which is promoted by Gq stimulation of phospholipase C. LPA stimulation of native HeLa cells (Figure 12, HeLa) resulted in a small 1.9-fold increase in accumulation of [3H] inositol phosphates, whereas stimulation of LPA₁/HeLa cells resulted in a large increase in PI hydrolysis (~14-fold) (Figure 12, lane 1).
Figure 12. Stimulation of phosphoinositide hydrolysis by LPA₁ receptors is inhibited by cholesterol extraction with methyl-β-cyclodextrin. Either native HeLa cells or stably transfected LPA₁/HeLa cells were labeled overnight with [3H] myo-inositol and then either left untreated (HeLa and lane 1) or pre-incubated with 5 mM MβCD for 1 hour (lane 2), 50 µg/ml nystatin for 1 hour (lane 4), 5 mM MβCD for 1 hour followed by 10 mM cholesterol/MβCD complexes for 60 minutes (lane 3), or 50 µg/ml nystatin for 1 hour followed by 10 mM cholesterol/MβCD complexes for 60 minutes (lane 5) prior to an additional 1 hour treatment with 10 µM LPA. Cells were then solubilized and the total accumulation of labeled inositol phosphates was determined. The radioactivity recovered in the different samples was normalized to total cellular protein and the data are presented as the mean±s.e.m. of triplicate measurements from a representative experiment that was repeated four times. **a, \( P<0.01 \), comparison of LPA-stimulated phosphoinositide hydrolysis in MβCD-treated LPA₁/HeLa cells to that observed in non-MβCD-treated LPA₁/HeLa cells. **b, \( P<0.01 \), comparing phosphoinositide hydrolysis in MβCD-treated or nystatin-treated LPA₁/HeLa cells that were incubated with water-soluble cholesterol to that observed in unstimulated LPA₁/HeLa cells.
Figure 13. Stimulation of phosphoinositide hydrolysis by LPA₁ but not M₁ mAChRs receptors is inhibited by cholesterol extraction with methyl-β-cyclodextrin. HeLa cells were transiently transfected with plasmids encoding either vector alone (lanes 1 and 2), LPA₁ (lanes 3 and 4), or M₁ mAChRs (lanes 5 and 6). The cells were incubated in the absence (−) or presence (+) of 5 mM MβCD for 1 hour prior to a subsequent 1 hour incubation with agonist (10 μM LPA or 1 mM carbachol). After solubilization, the radioactively labeled inositol phosphates were isolated as described. The radioactivity recovered in the different samples was normalized to total cellular protein and the data are presented as the mean±s.e.m. of triplicate measurements from a representative experiment that was repeated three times. **P<0.01, comparison of LPA-stimulated phosphoinositide hydrolysis in MβCD-treated LPA₁-transfected HeLa cells to that observed in non-MβCD-treated cells.
Table 1. Effects of cholesterol perturbing agents on cellular cholesterol abundance

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol content (µg/mg protein)*</th>
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<tbody>
<tr>
<td>Control</td>
<td>17.1±0.13</td>
</tr>
<tr>
<td>5 mM MβCD</td>
<td>6.5±0.05</td>
</tr>
<tr>
<td>5 mM MβCD + 10 mM cholesterol/MβCD</td>
<td>34.2±0.78</td>
</tr>
<tr>
<td>50 µg/ml Nystatin</td>
<td>20.8±1.5</td>
</tr>
<tr>
<td>50 µg/ml Nystatin + 10 mM cholesterol/MβCD</td>
<td>42.9±1.3</td>
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*Stably transfected LPA1/HeLa cells were left untreated (Control) or were incubated with 5 mM MβCD for 1 hour, 50 µg/ml nystatin for 1 hour, 5 mM MβCD for 1 hour followed by 10 mM cholesterol/MβCD for 1 hour or 50 µg/ml nystatin for 1 hour followed by 10 mM cholesterol/MβCD for 1 hour. Cells were then solubilized and total cellular cholesterol content was quantified as described in the Materials and Methods. The amount of cellular cholesterol was normalized to total cellular protein and is presented as the mean±s.e.m. of triplicate measurements from a representative experiment that was repeated twice.
Treatment of LPA₁/HeLa cells with 5 mM MβCD reduced LPA stimulated PI hydrolysis to 3.9-fold (72% inhibition) (Figure 12, lane 2). Treatment of LPA₁/HeLa cells with 50 µg/ml nystatin did not significantly affect agonist-dependent PI hydrolysis (Figure 12, lane 4). Addition of water-soluble cholesterol:MβCD complexes, which contained 10 mM cholesterol, to MβCD-treated or nystatin-treated cells greatly increased the extent of basal accumulation of labeled inositol phosphates (Figure 12, lanes 3 and 5). Addition of water-soluble cholesterol led to a greater increase in the basal level of inositol phosphate accumulation as compared to that observed in LPA stimulated samples, which consequently decreased the fold induction of PI hydrolysis by LPA. This could be due to enhanced Gαq signaling that is independent of LPA₁ receptors. These results suggested that the presence of membrane cholesterol was important for LPA₁ stimulation of PI hydrolysis. As a control, we examined the effects of MβCD on the stimulation of PI hydrolysis by an unrelated Gq-coupled receptor, the M1 muscarinic acetylcholine receptor (M1 mAChR). For these experiments, HeLa cells were transiently transfected with plasmids encoding either wild-type LPA₁ or M1 mAChRs. Whereas 5 mM MβCD inhibited LPA₁-mediated PI hydrolysis (Figure 13, compare lanes 3 and 4) as expected, it did not significantly reduce the extent of PI hydrolysis in response to agonist stimulation (1 mM carbachol) of M1 mAChR-expressing cells (Figure 13, compare lanes 5 and 6). Immunofluorescence microscopy indicated that the transfection efficiencies of the LPA₁ and M1 mAChR plasmids were comparable and were approximately 40% (data not shown). These results indicated that the reduction of LPA₁-mediated PI hydrolysis by MβCD was not due to inhibition of either Gαq or phospholipase C activities, but rather was due to a specific inhibition of LPA₁ function. Taken together, these results suggest
that the presence of plasma membrane cholesterol is critical for LPA₁-dependent signaling to phospholipase C (Figure 12).

Next, we investigated whether membrane cholesterol was also important for LPA₁ endocytosis. We compared the effects of MβCD on the agonist-induced endocytosis of LPA₁ and M1 mAChRs, which are also internalized by clathrin- and β- arrestin-dependent mechanisms (196). Stably transfected LPA₁/HeLa cells or transiently-transfected HeLa cells expressing M1 mAChRs were pre-incubated in the presence or absence of 5 mM MβCD for 1 hour. These cells were then incubated with the respective agonists and Alexa 594-Tfn for 30 minutes. In untreated cells, LPA₁ and M1 mAChRs localized to the plasma membrane, whereas Alexa 594-Tfn labeled pleomorphic endosomal structures (Figure 14, untreated). Agonist stimulation induced the endocytosis of both receptors into endosomal structures. To quantify GPCR endocytosis, we measured the extent of GPCR (either LPA₁ or M1) co-localization with the internalized Alexa 594-Tfn using Metamorph image analysis (186, 188). In control cells, treatment with 10 µM LPA increased LPA₁ and Alexa 594-Tfn co-localization by 3.3-fold relative to untreated cells (Figure 14, Control). Preincubation with 5 mM MβCD reduced this agonist-induced co-localization by more than 50% (Figure 14, compare black bars in control cells to cells treated with 5 mM MβCD). Addition of 10 mM water-soluble cholesterol restored LPA₁ and Alexa 594-Tfn co-localization to near control levels (Figure 14, MβCD + cholesterol). In contrast to LPA₁, agonist treatment (1 mM carbachol, 30 minutes) stimulated a twofold increase in M1 mAChR colocalization with Alexa 594-Tfn, which was not inhibited by MβCD (Figure 14). These data suggested that plasma membrane cholesterol is important for both LPA₁ endocytosis and signaling.
Figure 14. Cholesterol extraction inhibits the agonist induced endocytosis of LPA₁ but not M₁ mAChRs. (A) HeLa cells were transiently transfected with plasmids encoding either LPA₁ or M₁ mAChRs and were incubated in the absence (Untreated) or presence of agonist (10 µM LPA or 1 mM carbachol, respectively) and 50 µg/ml Alexa 594-Tfn for 30 minutes and then processed for indirect immunofluorescence localization of the transfected receptors. Bar, 10 µm. (B and C) HeLa cells were transfected as described above and were preincubated for 1 hour in the absence (Control) or presence of 5 mM MβCD or MβCD and 10 mM cholesterol/MβCD complexes prior to a subsequent incubation in the presence or absence of 10 µM LPA and 50 µg/ml Alexa 594-Tfn. The cells were fixed and processed for immunofluorescence localization of the transfected receptors. The extent of colocalization between LPA₁ (B) or M₁ mAChRs (C) and the internalized Alexa 594-Tfn was quantified using Metamorph image analysis as described in Materials and Methods. The data are expressed as the mean±s.e.m. of 20 cells/condition from a representative experiment that was performed three times with similar results. **P<0.01 compared with control, LPA-treated cells that colocalized with internalized Alexa 594-Tfn (Figure 14, +agonist).
4.4 LPA<sub>1</sub> localizes to detergent-resistant membrane microdomains upon agonist stimulation

Although the data above indicates that membrane cholesterol is essential for LPA<sub>1</sub> endocytosis, the data in Figure 8-11 indicate that LPA<sub>1</sub> is internalized by clathrin- and β-arrestin-dependent mechanisms, which are distinct from cholesterol-dependent endocytic pathways (26). To further investigate this apparent difference, we examined whether LPA<sub>1</sub> localized to detergent-resistant membrane domains, which are enriched in both cholesterol and glycosphingolipids (27). We examined the effects of LPA stimulation on the resistance of LPA<sub>1</sub> to extraction with Triton X-100. LPA<sub>1</sub>/HeLa cells were incubated with 10 µM LPA for different times before extraction with ice-cold 1% Triton X-100 and indirect immunofluorescence (Figure 15A). LPA<sub>1</sub> staining in unstimulated cells was greatly reduced following Triton X 100 extraction (Figure 15A, 0 minute). In contrast, the cell-associated LPA<sub>1</sub> staining, which remained after detergent extraction, was increased with time of agonist stimulation (Figure 15A). We quantified the detergent-resistant LPA<sub>1</sub> staining associated with the cells by measuring the pixel intensity of LPA<sub>1</sub>-specific fluorescence using MetaMorph image analysis (see materials and methods) and normalizing this value to DNA content as assessed by Hoechst dye labeling (Figure 15B). This analysis showed that detergent extraction of unstimulated cells reduced the level of cell-associated LPA<sub>1</sub> staining to 5% of that observed in untreated and non-extracted cells. Cell-associated LPA<sub>1</sub> staining progressively increased with time of LPA treatment such that after 30 minutes of LPA stimulation approximately 38% of LPA<sub>1</sub> immunoreactive staining remained after detergent extraction, relative to control cells (Figure 15B, solid circles). Extraction of membrane cholesterol with 5 mM MβCD
prior to LPA stimulation blocked the LPA-induced increase in detergent resistance of LPA₁ (Figure 15B, open triangles). As detergent resistance of proteins can also be increased by their association with the actin cytoskeleton, we examined the effects of inhibiting actin polymerization with cytochalasin D (5 µM) on the detergent resistance of LPA₁ (Figure 15B, open squares). We observed no noticeable difference between the detergent resistance of LPA₁ in cells pre-treated with cytochalasin D and untreated cells, after brief exposure to LPA (i.e. 0 to 8 minutes). A slight delay in the rate of increase of LPA₁ detergent resistance was observed between 10 and 20 minutes of LPA treatment in cells that were pre-treated with cytochalasin D, but the extent of detergent resistance was the same in both untreated and cytochalasin D-treated cells after 30 minutes of LPA stimulation. These data indicate that LPA treatment promotes the association of LPA₁ with detergent resistant membranes and that this process is inhibited by cholesterol extraction. Given that LPA treatment for 30 minutes promotes the endocytosis of LPA₁ into transferrin receptor+ endosomes, it is likely that some of the detergent resistant LPA₁ receptors observed after longer LPA treatment reside in endosomes. Studies have shown that transferrin receptor+ endosomes are enriched in cholesterol (Hao et al., 2002). To investigate the effects of agonist stimulation on the detergent resistance of surface LPA₁ receptors, we labeled surface LPA₁ with mouse anti-FLAG antibodies on ice prior to detergent extraction (Figure 16C,D). The LPA₁ expressed in LPA₁/HeLa cells contains an N-terminal FLAG epitope tag that is accessible to the extracellular medium. In the absence of detergent, mouse anti-FLAG antibodies labeled only the cell surface in control cells (Figure 16C, Control). Triton X-100 extraction removed most of the surface-bound antibody (only 15% of control, un-extracted cells remained) (Figure 16C,D, 0
minute). The detergent resistance of surface LPA₁ increased with time of agonist treatment up to 45% of control levels after 6 minutes and then declined after 15 minutes and 30 minutes of LPA treatment (Figure 16C,D). These results suggest that the LPA₁ receptor associates with detergent-resistant membranes following agonist stimulation, both at the cell surface and following endocytosis in cholesterol-rich endosomes.
Figure 15. LPA₁ receptors localize to detergent-resistant cellular domains upon agonist stimulation (total fluorescence). A) LPA₁/HeLa cells were incubated with 10 µM LPA for different times and subsequently treated with 1% cold Triton X-100 on ice for 3 minutes, fixed and processed for indirect immunofluorescence localization of LPA₁. B) Quantitative analysis of receptor expression after detergent extraction was performed by MetaMorph image analysis as described in Materials and Methods. Cells were either untreated, treated with 5 µM cytochalasin D (Cyto. D) for 30 minutes, or treated with 5 mM MβCD for 1 hour, prior to incubation with 10 µM LPA for the indicated times. The LPA₁ labeling in detergent-extracted cells was normalized to the amount of LPA₁ labeling observed in non-agonist-treated cells, which had not been subjected to detergent extraction. The data are presented as the mean±s.e.m. of five to six cells per time point and are from a representative experiment that was repeated twice with similar results.
Figure 16. LPA₁ receptors localize to detergent-resistant cellular domains upon agonist stimulation (surface fluorescence). (C) LPA₁/HeLa cells were incubated with 10 µM LPA for different times and incubated with mouse anti-FLAG antibody on ice for 30 minutes prior to extraction with ice-cold 1% Triton X-100, to label surface LPA₁ receptors. Cells were then processed for indirect immunofluorescence localization of surface LPA₁. (D) Quantitative analysis of surface LPA₁ receptor expression after detergent extraction was performed by MetaMorph image analysis as described in Materials and Methods. The LPA₁ labeling in detergent extracted cells was normalized to the amount of LPA₁ labeling observed in non-agonist treated cells, which had not been subjected to detergent extraction. The data are presented as the mean±s.e.m. of five to six cells per time point and are from a representative experiment that was repeated twice with similar results. **P<0.01, comparison of the amount of detergent-resistant surface LPA₁ staining after the indicated time of agonist treatment with that observed in unstimulated cells. Bar, 10 µm.
4.5 Membrane cholesterol is required for the plasma membrane recruitment of cytosolic β-arrestins by activated LPA₁

As β-arrestins are required for the clathrin-mediated endocytosis of LPA₁, we examined whether membrane cholesterol was important for the association of β-arrestins with LPA₁ or with β2AR, as a control. Preliminary experiments showed that, in HeLa cells that were transiently transfected with plasmid encoding either LPA₁ or β2ARs along with β-arrestin -2-GFP, both LPA₁ and β2ARs transiently recruited cytosolic β-arrestin-2-GFP to punctate plasma membrane structures after 2 minutes of agonist stimulation (Figure 18, Control). Double-labeling experiments showed that β-arrestin-2-GFP extensively co-localized with the plasma membrane clathrin adaptor, AP2 (Figure 17), suggesting that brief LPA stimulation led to the recruitment of β-arrestin-2-GFP to cell surface clathrin-coated pits. After 30 minutes of agonist stimulation, both LPA₁ and β2ARs localized to endosomal structures, but β-arrestin 2 GFP returned to a cytosolic distribution (data not shown). This is consistent with published reports showing that β2ARs transiently associate with β-arrestins (19).

We next examined the effects of cholesterol extraction on the surface recruitment of β-arrestin-2-GFP by LPA₁ and β2AR after 2 minutes of agonist stimulation (Figure 8). β-arrestin-2- GFP localized in a diffuse cytoplasmic pattern in unstimulated cells and both LPA₁ and β2AR were localized to the plasma membrane (data not shown). After 2 minutes of LPA treatment, β-arrestin-2-GFP colocalized with LPA₁ in punctate spots at the cell surface (Figure 18, Control, left panels), which also colocalized with AP2 (see Figure 17).
Figure 17. LPA stimulation leads to the co-localization of \( \beta \)-arrestin-2-GFP with clathrin AP2 adaptors. LPA\(_1\)/HeLa cells were transiently transfected with plasmids encoding \( \beta \)-arrestin-2-GFP and incubated with 10 \( \mu \)M LPA for either 0 minute or 2 minutes prior to fixation. Endogenous clathrin AP2 was localized in permeabilized cells using mouse anti-AP2 antibodies. The inset shows a high magnification image of the boxed region and the arrows indicate structures where \( \beta \)-arrestin-2-GFP co-localized with AP2. Bar, 10 \( \mu \)m.
Similarly, after 2 minutes of isoproterenol treatment of β2AR-expressing cells, β-arrestin-2-GFP localized to punctate spots at the cell surface (Figure 18, Control, right panels). Pre-incubation with 5 mM MβCD for 60 minutes completely inhibited the recruitment of β-arrestin-2-GFP to the cell surface by LPA₁ and β-arrestin-2-GFP remained in a diffuse cytosolic distribution (Figure 18, MβCD, see inset). Addition of 10 mM water-soluble cholesterol restored the ability of LPA₁ to recruit β-arrestin-2-GFP to the cell surface in MβCD-treated cells (Figure 18, MβCD:cholesterol). In contrast, incubation with MβCD did not inhibit β-arrestin-2-GFP recruitment to punctate surface spots by β2ARs (Figure 18, MβCD, see inset). Addition of water-soluble cholesterol did not alter the surface recruitment of β-arrestin 2-GFP by β2ARs (Figure 18, MβCD-cholesterol). To quantify these effects, we determined the percentage of cells that showed β-arrestin recruitment to the plasma membrane after 2 minutes of agonist stimulation of either LPA₁ or β2AR (Figure 19). In control cells and in cholesterol repleted cells (i.e. MβCD-cholesterol), β-arrestin-2-GFP was recruited to the cell surface in ~80% of cells expressing either LPA₁ or β2AR. Only 4% of LPA₁-expressing cells showed surface recruitment of β-arrestin-2-GFP in MβCD-treated cells. In contrast, approximately 50% of β2AR-expressing cells exhibited surface recruitment of β-arrestin-2-GFP. Taken together, these results indicate that LPA₁ is much more dependent upon plasma membrane cholesterol for the recruitment of β-arrestin than β2ARs. This also provides a possible link between membrane cholesterol and clathrin dependent endocytosis of LPA₁ as β-arrestin is critical for endocytosis of LPA₁.
Figure 18. mβcd extraction prevents recruitment of β-arrestin-2-GFP to the plasma membrane by LPA₁ but not by β₂AR. (a,b) HeLa cells were transiently transfected with plasmid encoding either LPA₁ (a) or β₂AR (b) along with β-arrestin-2-GFP. The cells were then left untreated, pretreated with 5 mM mβcd for 1 hour, or treated sequentially with 5 mM mβcd for 1 hour and 10 mM water-soluble cholesterol for 1 hour prior to incubation with 10 μM LPA for 2 minutes. The cells were then fixed and processed for indirect immunofluorescence microscopy. The inset shows a magnified image of the region of the cell indicated by the arrow.
Figure 19. Phenotypic quantitation of recruitment of β-arrestin-2-GFP to the plasma membrane. The percentage of cells exhibiting recruitment of β-arrestin-2-GFP to punctate plasma membrane spots after 2 minutes agonist stimulation was determined by scoring 100 cells per condition for cells expressing LPA₁ and β₂AR. Bar, 10 μm.
CHAPTER 5
DISCUSSION

All members of the GPCR superfamily share the ability to rapidly respond to agonist stimulation and then to undergo desensitization (181). Many of these GPCRs are also rapidly internalized into cells via one of several distinct endocytic pathways. However, the mechanisms that regulate GPCR desensitization and determine the specific endocytic pathway used for internalization vary from receptor to receptor. In this study, we found that LPA₁ receptors are internalized by a clathrin- and β-arrestin-dependent pathway, but that they also require plasma membrane cholesterol for receptor signaling and for their subsequent clathrin-dependent endocytosis. Our results indicate that the key requirement of membrane cholesterol for LPA₁ endocytosis is for the association of LPA₁ with β-arrestin, which promotes both signal attenuation and clathrin-dependent endocytosis of the receptor.

Caveolae and other detergent-resistant membrane domains are cholesterol-and glycosphingolipid-rich, are sites of active signal transduction and have been implicated in the activation of heterotrimeric G proteins, Ras signaling and eNOS signaling (27). Several lines of evidence suggest that LPA₁Rs associate with cholesterol-rich, detergent-resistant membranes and that this is important for LPA₁-dependent signaling. First, cholesterol extraction with MβCD strongly inhibited LPA₁ induction of phosphoinositol hydrolysis, via Gαq-mediated stimulation of phospholipase C (Figure 12). Gαq has been shown to be enriched in caveolae (197, 198), which supports the notion that LPA₁ stimulates PI hydrolysis by associating with Gαq in detergent resistant membranes. Re-
addition of cholesterol to MβCD treated cells increased both the basal and LPA-stimulated levels of PI hydrolysis (Figure 12). The fact that MβCD extraction did not affect PI hydrolysis by the M1 mAChR suggests that cholesterol depletion does not impair either Gαq or phospholipase C activity per se, but that LPA₁ stimulation of PI hydrolysis is particularly sensitive to cholesterol depletion (Figure 13). Two possible explanations for the difference between LPA₁ and M1 mAChRs are that either LPA₁ exclusively couples to Gαq that is localized to detergent-resistant membrane domains or that membrane cholesterol is required for the physical association of LPA₁ with Gαq.

Second, we found that LPA stimulation enhanced the resistance of both surface and total LPA₁ to extraction with TX-100 detergent (Figure 15). Resistance to detergent extraction is a common property of proteins that are associated with caveolae and other cholesterol-rich membrane regions (27). The detergent resistance of surface LPA₁ increased during the first 6 minutes of LPA treatment and then declined. This is consistent with a transient association of LPA₁ with detergent-resistant microdomains prior to β-arrestin- and clathrin-dependent endocytosis. Disruption of the actin cytoskeleton with 5 µM cytochalasin D did not alter the agonist-induced detergent resistance of LPA₁ suggesting that the increased detergent resistance of LPA₁ was not due to its association with the actin cytoskeleton. However, cholesterol extraction completely prevented the agonist-induced detergent resistance of LPA₁, which is consistent with the notion that LPA₁ associates with cholesterol-rich membrane microdomains.

Interestingly, total detergent-resistant LPA₁ staining increased with time of LPA treatment even after longer periods of agonist stimulation (Figure 15B, 30 minutes). We have previously shown that about 35-40% of surface LPA₁ receptors are internalized into
transferrin receptor+ endosomes after 30 minutes of LPA treatment (186). We hypothesize that some of the detergent-resistant LPA1 staining observed after longer LPA treatment resides in transferrin receptor+ endosomes, which are known to be enriched in cholesterol (199). Finally, a recent study showed that LPA stimulation of phosphoinositide 3-kinase and the downstream effector kinase, Akt, was inhibited by MβCD treatment in Vero cells (184). Collectively, these data suggest that LPA1 association with cholesterol-rich plasma membrane regions is critical for LPA-induced signaling through Gαq. Given that a pool of Gαq is present in cholesterol-rich caveolae, we hypothesize that the localization of LPA1 to detergent resistant membranes is important for their association with the pool of Gαq that is localized to these domains.

In support of a role for cholesterol in LPA1 endocytosis, we found that cholesterol extraction inhibited LPA1 association with β-arrestin and the subsequent clathrin-dependent endocytosis of the receptor. Many different GPCRs interact with β-arrestins, which is important for the proper regulation of receptor function (181). Phosphorylation of specific serine/threonine residues in either the cytoplasmic tail or the third intracellular loop, by G protein receptor kinases, leads to the recruitment of β-arrestin proteins, which in turn block G protein/receptor coupling (desensitization) and also promote clathrin-dependent endocytosis (22). Our data show that wild-type LPA1 receptors transiently recruit β-arrestin-2-GFP to discrete AP2+ structures at the cell surface, in an agonist-stimulated fashion (Figure 17), but do not co-localize on endosomes with β-arrestin-2-GFP (Figs 14 and 18). β-arrestins promote clathrin-dependent endocytosis of GPCRs by localizing receptors to clathrin coated pits through an interaction of β-arrestins with both clathrin heavy chain and the μ2 subunit of the AP-2 clathrin adaptor complex (23, 200).
Using MEFs derived from β-arrestin 1 and 2 double-knockout mice, we showed that both signal attenuation and endocytosis of LPA₁ is dependent upon β-arrestin (Figs 9, 10 and 11). Using an RNA interference approach to reduce the cellular abundance of clathrin heavy chain, we showed that knockdown of clathrin inhibited the internalization of LPA₁, transferrin receptors, but not the internalization of the GPI-anchored protein, CD59, which localize to cholesterol rich membrane regions (Figure 8). Taken together, these data indicate that LPA₁ receptors are internalized by β-arrestin- and clathrin-dependent endocytosis.

The most significant finding of these studies was that cholesterol extraction inhibited β-arrestin recruitment to the plasma membrane by LPA₁ and the subsequent endocytosis of these receptors (Figs 14 and 18) and that re-addition of cholesterol to MβCD treated cells restored both of these functions. As β- arrestin binding to LPA₁ precedes receptor endocytosis, we hypothesize that cholesterol is required for the association of LPA₁ with β-arrestins and that it is the lack of β-arrestin binding that leads to the inhibition of LPA₁ endocytosis. This is a novel and previously unappreciated role for membrane cholesterol in the recruitment of β-arrestins. We speculate that other GPCRs that localize to caveolae may also associate with β-arrestin in a cholesterol-dependent manner. β2ARs localize to caveolae in cardiomyocytes in the absence of agonist but move out of caveolae and into clathrin-coated pits upon ligand binding (30, 201). As β2AR endocytosis requires β-arrestin binding, it is probable that β-arrestin also binds to these receptors in caveolae. Whether the association of β-arrestin with β2ARs in cardiomyocytes is cholesterol dependent remains to be determined.
In contrast to LPA$_1$, cholesterol extraction did not inhibit the endocytosis of M1 mAChRs (Figure 13), which also follow a $\beta$- arrestin- and clathrin-dependent pathway (196) nor did cholesterol extraction inhibit the association of $\beta$2ARs with $\beta$-arrestins. This suggests that the cholesterol dependence of $\beta$-arrestin recruitment is a unique property of LPA$_1$. Cholesterol may be important either for the direct recruitment and binding of $\beta$-arrestins to LPA$_1$Rs or for the recruitment of kinases such as GRKs that phosphorylate agonist-stimulated LPA$_1$. Indeed, GRK4 and GRK6 are palmitoylated, which is required for their membrane association (202, 203), and palmitoylation has been shown to target many proteins to cholesterol-rich membranes including SNARES (204), flotillins (205) and RGS16 (206). Recent work has shown that both LPA stimulation and activation of protein kinase C with phorbol esters promotes LPA$_1$ phosphorylation (207).

Are there physiological contexts where changes in cellular cholesterol modulate LPA signaling? One intriguing example may be prostate cancer cells, whose growth is potently stimulated by LPA. Cholesterol is elevated in these cells and LPA$_1$ receptor signaling and trafficking contributes to their enhanced growth (88, 208, 209), perhaps by augmenting LPA signaling. Future studies should provide the answer to this and other questions about this novel process.
CHAPTER 6
FUTURE DIRECTIONS

In the previous chapter we had shown that membrane cholesterol is required for LPA-dependent localization of the LPA₁ receptor to detergent resistant membrane microdomains in a time-dependent manner. Membrane cholesterol was required for LPA-induced, Gαq-mediated phosphoinositide hydrolysis and subsequent endocytosis. We had also shown that membrane cholesterol is not required for LPA-induced, Gαi-mediated MAPK activation (unpublished observations). Together, these data would suggest that LPA-induced signaling from the LPA₁ receptor to different G-proteins might be localized to different microdomains of the plasma membrane. Thus compartmentalization of signaling could potentially lead to specific targeting of a signaling pathway, eventually leading to regulation of a specific physiological response. Further studies need to be done to determine if the LPA₁ receptor associates with Gαi in detergent sensitive membrane microdomains and if this association occurs before the LPA₁ receptor localizes to detergent resistant microdomains (3 minutes). An important question that arises from the results in the previous chapter is what is the requirement for the localization of the LPA₁ receptor into detergent resistant membranes? One hypothesis is that the GRK, which is required to phosphorylate the LPA₁ receptor, localizes to DRMs, thus leading to cholesterol-dependent β-arrestin recruitment, signal desensitization, and internalization. It is known that GRK4 or GRK6 can be palmitoylated causing them to localize to detergent resistant microdomains. Further studies need to be done to determine if this hypothesis is true or not.
PART II:

DIFFERENT MOTIFS ARE REQUIRED FOR AGONIST-DEPENDENT VERSUS AGONIST-INDEPENDENT ENDOCYTOSIS OF THE LPA₁ LYSOPHOSPHATIDIC ACID RECEPTOR
CHAPTER 7

INTRODUCTION

The serum phospholipid Lysophosphatidic acid (LPA) has growth-factor like properties and is involved in a variety of processes like wound healing, cell proliferation and survival, neurite retraction and cell migration (121). The diversity of responses elicited by LPA has been attributed to the fact that it mediates its effects through multiple G-protein coupled receptors (GPCRs). LPA has been shown to activate five GPCRs termed LPA_{1-5} (116, 117). Multiple variations in the domains of GPCRs give rise to the myriad of GPCRs found in different organisms, which differ in both structure and function. The plasma membrane expression of functional GPCRs can be regulated by a variety of mechanisms such as endocytosis, endosomal sorting, recycling and degradation. These mechanisms are regulated via a range of interactions mediated by motifs in the cytoplasmically exposed domains of GPCRs depending on their respective structure (1, 13, 210). Many GPCRs utilize either a clathrin-dependent pathway or one of a number of clathrin-independent pathways for internalization (211-214). Adaptor proteins involved in mediating endocytosis of GPCRs via a clathrin-dependent pathway include Clathrin, AP-2, and β-arrestins, which sort receptors into clathrin coated pits (CCPs) (1, 19, 23).

β-arrestins have been shown to interact with many GPCRs (β2AR, LPA_{1}) (1, 214) and have been shown to bind to serine/threonine residues, which have been preferentially phosphorylated by G-protein Receptor kinases (GRKs) (1). Interestingly, GPCRs like the thrombin activated PAR-1 do not require β-arrestins to mediate endocytosis (182).
Additionally, many GPCRs have been shown to be phosphorylated by second messenger kinases like PKA and PKC, primarily leading to desensitization or in some cases internalization (15, 215, 216). Association of GPCRs with β-arrestins typically causes signal desensitization followed by receptor endocytosis, where endocytosis is mediated by a direct interaction with members of the endocytic machinery, AP-2 and Clathrin (1, 23). Depending on the characteristics of agonist-dependent arrestin association, two classes of GPCRs have been proposed: Class A and Class B, which differ in their affinities for β-arrestin 1 and 2 and in their temporal-spatial association (31).

Apart from β-arrestins, other adaptor proteins like AP-2 have been shown to mediate internalization of receptors in a β-arrestin independent fashion (PAR-1, TA2) (33, 182, 217). Several studies have shown that certain motifs like the dileucine- (LL/IL) and tyrosine-based (YXXφ) motifs in the cytoplasmic tails of GPCRs can mediate direct interactions with AP-2, facilitating endocytosis (33, 53, 54). Dileucine motifs have been found in the cytoplasmic tails of GPCRs and can mediate both agonist-dependent and agonist-independent internalization (218, 219).

We have previously shown that the LPA1 receptor utilizes a clathrin - and β-arrestin – dependent pathway for internalization following agonist treatment (186, 214). In this study we sought to investigate the motifs in the cytoplasmic tail of the LPA1 receptor that might be involved in the internalization of the receptor. Two distinct motifs in the cytoplasmic tail are shown to be required for agonist-dependent versus agonist-independent internalization of the LPA1 receptor. A serine cluster in the tail is required for transient β-arrestin association mediating agonist-dependent signal desensitization as well as subsequent endocytosis. However, a distal dileucine motif is required for agonist-
independent internalization of the LPA$_1$ receptor, which appears to be β-arrestin
independent, AP-2 dependent and protein kinase C (PKC) dependent. This suggests that
the LPA$_1$ receptor utilizes two distinct mechanisms for agonist-dependent versus agonist-
independent internalization.
8.1 Antibodies and reagents

Lysophosphatidic acid (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate; LPA) was purchased from Avanti Polar Lipids (Alabaster, AL). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Co. (St Louis, MO). Wild-type and mutant HA-tagged LPA₁ receptors were detected with mouse anti-HA antibodies (Covance, Berkeley, CA). myo-[3H]inositol was purchased from American Radiolabeled Chemicals (St Louis, MO); Bisindolylmaleimide I was purchased from Calbiochem. Cy3 Donkey anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch.

8.2 Plasmids

HA-LPA₁ plasmid was generated by PCR, using a previously described FLAG-LPA₁ plasmid as template (183). HA-tagged truncation mutant receptors, as described in the figure legends, were generated using the Gene-Tailor site-directed mutagenesis kit (Invitrogen, Carlsbad, CA), according to manufacturer’s instructions, with the HA-LPA₁ wild-type plasmid as template. All plasmids were subjected to DNA sequencing to confirm their sequences. β-arrestin GFP plasmids were kindly provided by Dr. Stefano Marullo.
8.3 Cell culture and DNA transfection

HeLa cells stably expressing HA-tagged LPA1 receptor (termed LPA1/HeLa cells), native HeLa cells, wild-type (WT) MEF and β-arrestin 1/2 KO MEF cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin (Media Tech, Herndon, VA) and 1 mM sodium pyruvate (Biosource International, Camarillo, CA) at 37°C with 5% CO2. Cells were grown on glass coverslips (for immunofluorescence) and transfected in six-well dishes, or were grown in 24-well dishes (for myo-[3H]inositol labeling) and transfected using Exgen 500 (Fermentas) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer’s directions.

8.4 Internalization Assay

LPA1/HeLa cells or HeLa cells were plated on coverslips. For internalization assays involving different mutant LPA1 receptors (Figure 6), HeLa cells were transfected with plasmid DNA as described in the figure legends. For experiments with siRNA-mediated reduction of AP-2 (Figure 8), LPA1/HeLa cells were transfected with siAP2 as described in the figure legends. The day before experimentation, the cells were serum starved overnight. Prior to any acute treatments, cells were transferred onto ice and incubated with mouse anti-HA primary antibodies (Covance) for 1hr to label cell surface HA tagged-LPA1 wild-type and mutant receptors. Cells were then either left at 4°C (total surface) or transferred to 37°C and treated as described in the figure legends. Following treatments at 37°C, antibodies bound to the cell surface were removed by rinsing the cells with 100 mM glycine, 20 mM magnesium acetate, 50 mM KCl, pH 2.2 (acid wash) (187)
for 90 seconds, while retaining internalized antibody. For experiments with co-
transfected HA-LPA$_1$ receptors and β-arrestin2 GFP (Figures 5 and 6), the surface
antibodies were not removed by acid wash. Cells were rinsed and processed for
immunofluorescence as described below.

8.5 Immunofluorescence

HA- LPA$_1$ receptors were detected using mouse anti-HA antibody (Covance). AP-2 was
detected using a mouse anti-AP2 antibody (AP.6) (Santacruz Biotechnology). For
internalization assays, following anti-HA antibody incubations at 4°C, treatments at
37°C, and acid wash, the cells were then fixed in 2% formaldehyde in phosphate buffered
saline (PBS) for 10 minutes and rinsed with 10% fetal bovine serum (FBS) containing
0.02% azide in PBS (PBS-serum). For AP-2 detection, following overnight serum
starvation, cells were fixed and rinsed with PBS-serum and were incubated with mouse
anti-AP2 antibodies diluted in PBS serum containing 0.2% saponin for 45 minutes.
Following fixation and incubation with primary antibodies, the cells were washed three
times with PBS serum and then incubated with fluorescently labeled donkey anti-mouse
secondary antibodies (Jackson ImmunoResearch) diluted in PBS-serum containing 0.2%
saponin for 45 minutes, washed three times with PBS-serum, washed once with PBS and
mounted on glass slides as previously described (183). Images were captured using
Hamamatsu digital camera mounted on a Leica Inverted microscope with a 63X oil
immersion objective. Images were processed with Adobe Photoshop 7.0 software.
8.6 Quantitation of LPA₁ Internalization

For internalization assays, images were taken using a Hamamatsu digital camera mounted on a Leica Inverted microscope with a 63X oil immersion objective. The images were analyzed by Simple PCI software (Compix, Cranberry Township, PA) and total fluorescence (vesicles/cell) for both internalized and surface antibody levels were measured as described previously (220). Internalization (fluorescence after acid wash) is expressed as a percentage of total fluorescence of initial surface bound antibodies (4°C) for both wild-type and mutant LPA₁ receptors.

8.7 siRNA-mediated reduction of AP-2

siRNA oligonucleotides to the µ-subunit of adaptin were purchased from Dharmacon (Lafayette, CO) and have been described previously (185). LPA1/HeLa cells were transiently transfected with 175 pmol (12-well plate) or 300 pmol (6-well plate) of siRNA to AP-2 (siAP2) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection medium was replaced with complete medium (without penicillin/streptomycin) 5 hours later and the cells were trypsinized after 2 hours and plated in a 6-well plate with (immunofluorescence) or without (immunoblotting) coverslips and then incubated for 16 hours. The cells were transfected a second time as above and the medium was then replaced with serum-free medium (SFM) and incubated for an additional 16 hours before experimentation.
8.8 Immunoblotting

Following serum starvation overnight, cells were either incubated on ice with mouse anti-HA antibodies for 30 minutes to label surface LPA₁ receptor molecules (for phosphoinositide hydrolysis) and/or were solubilized by addition of lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 M sodium orthovanadate, 0.02% azide, 100 µg/ml leupeptin and 0.1 mM PMSF) and incubated on ice for 60 minutes. The samples were then separated by 10% SDS-PAGE and transferred to nitrocellulose. The nitrocellulose membrane was incubated with mouse anti-HA (HA- LPA₁ receptor) (Covance) or mouse anti-AP2 (for detection of AP-2) (Santacruz Biotechnology) antibodies. The binding of primary antibodies was detected by using the West Pico enhanced chemiluminescence detection kit (Pierce Biotechnology, Rockford, IL).

8.9 Phosphoinositide hydrolysis

HeLa cells were plated at a density of 4 x 10⁴ cells/well into 24-well plates and transfected with plasmids encoding wild-type or mutant HA-LPA₁ using Exgen500 (Fermentas). At 24 hours post-transfection, cells were labeled overnight with myo-[³H]inositol in inositol- and serum-free medium, treated as described in the figure legends and then processed for analysis of phosphoinositide hydrolysis by anion exchange chromatography as described (214).
8.10 Statistical analysis

The data is expressed as the mean±s.e.m. from the indicated number of independent experiments performed in triplicate. Differences were analyzed by two-factor ANOVA followed by a Tukey’s statistical significance test.
CHAPTER 9

RESULTS

9.1 Agonist-independent internalization of the LPA₁ receptor is PKC-dependent

A previous study had suggested a role for PKC phosphorylation in the PMA-induced internalization of the LPA₁ receptor. PMA treatment caused desensitization and internalization of the LPA₁ receptor in C9 rat hepatoma cells, which was sensitive to PKC inhibitors (207). To determine whether PMA treatment caused internalization of the LPA₁ receptor in our LPA₁/HeLa cells, we treated the cells with 1µM PMA and used the PKC inhibitor Bisindolylmaleimide I (Bis I) to inhibit the effects of PMA treatment. To measure internalization we bound mouse-anti HA antibodies to the cells expressing the HA-tagged LPA₁ receptor at 4°C and then either left the cells at 4°C (total surface) or transferred these to 37°C. After the treatments at 37°C, the surface bound antibodies were removed with a mild acid wash (see materials and methods) while retaining the internalized antibody bound to the LPA₁ receptor. As shown in Figure 20, in the 4°C (–Bis I) untreated control cells (initial surface bound antibodies), the anti-HA antibodies bound to the LPA₁ receptor show a plasma membrane distribution. In the 37°C untreated cells (with acid wash) the anti-HA antibodies bound to the LPA₁ receptor show a predominantly internal/endosomal localization (Figure 20, -Bis I/37°C untreated), as determined by co-localization with Alexa 594-Transferrin (data not shown). This internal pool of receptor molecules represents basal internalization of the HA-tagged LPA₁ receptor and its levels are about 15% of the 4°C untreated (–BisI) control (Fig 21, 37°C Untreated/white bar).
Figure 20. Agonist independent internalization of the LPA₁ receptor is PKC-dependent.

For stably transfected LPA₁/HeLa cells, surface receptors were labeled with mouse anti-HA antibody (Covance) at 4°C for 1 hour. The cells were washed and were either left at 4°C or transferred to 37°C. The cells at 37°C were then treated with (+ Bis I) or without (- Bis I) Bisindolylmaleimide I for 30 min at 37°C, prior to incubation in the absence (untreated) or presence of either 10 μM LPA or 1 μM PMA for 30 min at 37°C. After the antibody-receptor complexes were allowed to internalize, the remaining surface bound antibodies were removed by mild acid wash. The cells at both 4°C and 37°C were then fixed and processed for immunofluorescence staining as described in materials and methods.
Figure 21. Quantitation of internalization of the LPA1 receptor.

Internalized antibody bound to the LPA1 receptor was quantified using Simple PCI software as described in materials and methods. Internalization was expressed as the percent of receptor associated antibody following acid wash, relative to the total fluorescence of surface bound antibody of the 4°C control. * p<0.05
After treatment with 10μM LPA for 30 minutes, the anti-HA antibodies bound to the LPA₁ receptor localized to punctate endosomal structures (Figure 20, -Bis I/ 37°C LPA) that have been previously shown to co-localize with transferrin-positive endosomes (183, 214). This internal pool of LPA₁ receptors after LPA treatment represents about 30% of the 4°C untreated (-Bis I) control (Figure 21, 37°C LPA/white bar). Treatment with 1μM PMA also led to a predominantly endosomal localization of the anti-HA antibodies bound to the LPA₁ receptor (Figure 20, -Bis I/ 37°C PMA), which represents ~22% of the 4°C untreated (-Bis I) control (Figure 21, 37°C PMA/white bar). A 30 minute pre-exposure with 5μM Bisindolylmaleimide I (+Bis I), a classical PKC inhibitor, blocked basal internalization (~60% reduction) (Figure 20, +BisI/ 37°C untreated and Figure 21, 37°C Untreated/black bar) as well as PMA-induced internalization (~80% reduction) (Figure 20, +Bis I/ 37°C PMA and Figure 21, 37°C PMA/black bar) of the mouse anti-HA antibodies bound to the LPA₁ receptor. Bis I pre-exposure did not completely inhibit LPA-induced internalization but reduced it to ~20% of the 4°C untreated (+ Bis I) control (Figure 20, +Bis I/37°C LPA and Figure 21, 37°C LPA/black bar). To confirm if antibody binding itself did not cause internalization of the LPA₁ receptor, we repeated the above experiments wherein the anti-HA antibodies were bound to the receptor after all acute treatments and fixing the cells in 2% PBS-formaldehyde. We observed a similar trend as the results in Figure 1 (data not shown). These results suggest that both PMA-induced and basal internalization are PKC-dependent and most likely follow a similar pathway of internalization, whereas inhibition of PKC has a partial effect on LPA-induced internalization.
9.2 A serine rich region in the tail of the LPA₁ receptor is required for signal desensitization

Clusters of serine residues on GPCRs have been shown to be important for agonist-dependent interaction with β-arrestins (51, 65). We had previously shown that the LPA₁ receptor forms a transient interaction with β-arrestins at the plasma membrane and is required for signal desensitization (214). The LPA₁ receptor has a serine cluster between amino acids 340 and 347, so we asked the question if this serine cluster is required for β-arrestin association and subsequent signal desensitization and internalization. We generated HA-tagged truncation mutant LPA₁ receptors as described in materials and methods. The HA-tagged wild-type and mutant receptors were functional as determined by a phosphoinositide hydrolysis assay (see materials and methods), which measures Gαq-dependent signaling upon LPA stimulation (Figure 23) (182, 214). We observed that in comparison to wild-type LPA₁ receptor, the LPA₁ Δ340 mutant showed elevated levels of accumulation of [H³] inositol phosphates in response to LPA stimulation, suggesting that it fails to desensitize Gαq signaling (Figure 23). In contrast, the Δ347 LPA₁ receptor showed reduced levels of [H³] inositol phosphate accumulation as compared to the Δ340 LPA₁ receptor, although compared to wild-type levels the Δ347 LPA₁ receptor still showed elevated [H³] inositol phosphate accumulation. The Δ353 LPA₁ receptor mutant showed further reduced levels of [H³] inositol phosphate accumulation compared to the Δ340 LPA₁ mutant receptor. The Δ361 mutant that lacks the PDZ binding domain showed reduced levels of [H³] inositol phosphate accumulation as compared to the wild-type LPA₁ receptor.
Figure 22. Schematic diagram of the LPA₁ receptor tail mutants

The uppermost sequence is the wild-type amino-acid sequence of the LPA₁ receptor tail describing the various motifs in the tail. The LPA₁ truncation mutants are shown with a Δ symbol, with their sequences ending in premature stop codons (*).
These results suggest that the region between residues 340-347 are critical for signal desensitization and therefore could also play a role in β-arrestin interaction and subsequent internalization of the LPA₁ receptor.

### 9.3 Different motifs in the tail of the LPA₁ receptor are required for LPA- versus PMA-dependent internalization

The results from Figure 23 suggested that the serine rich region between residues 340-347 might be critical for signal desensitization and therefore β-arrestin interaction and subsequent internalization. We next sought to determine whether the serine rich region was required for LPA-dependent internalization. HeLa cells were transiently transfected with different HA-tagged LPA₁ receptors as described in Figure 24 and were exposed to 10 µM LPA or 1 µM PMA for 30 minutes. We measured internalization of the HA-tagged WT and mutant LPA₁ receptors similar to Figure 21. In the 4°C untreated control cells expressing WT or mutant LPA₁ receptors, the mouse anti-HA antibody bound to the receptor showed predominant plasma membrane localization (data not shown). As in Figure 21, the internalization is expressed as a percentage of the initial antibody bound in the 4°C untreated control cells. The wild-type LPA₁ receptor internalized (16% of 4°C untreated control) in response to LPA stimulation (Figure 24, WT/LPA and Figure 25, WT/black bars), whereas the Δ340 LPA₁ receptor, which lacks the serine rich region, showed a ~75% reduction in internalization in response to LPA treatment (Figure 24, 340/LPA and Fig 25, 340/black bar).
HeLa cells were transiently transfected with plasmids encoding either wild-type LPA₁ (WT) or truncated mutant LPA₁ (Δ340-Δ361) receptors. The cells were processed as described in materials and methods, prior to incubation in the absence (Untreated) or presence (10μM LPA) of agonist for 1 hour. After solubilization, the radioactively labeled inositol phosphates were isolated as described previously (182). The radioactivity recovered in the different samples was normalized to both total cellular protein and total receptor surface expression as determined by western blot (data not shown). The data are presented as the mean±s.e.m. of triplicate measurements from a representative experiment that was repeated three times. * p<0.05 – compare LPA stimulated condition for Δ340 with Δ347 and Δ353.
In contrast, the Δ347, Δ353 and Δ361 mutants that have the serine rich region internalized comparable to wild-type levels (~12% of 4°C untreated control) (Figure 24, and Figure 25, 347,353,361/black bars). In addition to LPA stimulation, we also tested the effects of PMA treatment on the different mutant LPA₁ receptors. The wild-type receptors internalized (~10% of 4°C untreated controls) in response to 1μM PMA treatment for 30 minutes (Fig 24 and Figure 25, WT/grey bar). In contrast to LPA treatment, both Δ340 and Δ347 failed to internalize upon PMA treatment (Fig 24 and Figure 25, 340,347/grey bar). Interestingly, Δ353 and Δ361 both of which have a dileucine motif, internalized in response to PMA treatment (Fig 24 and Figure 25, 353,361/grey bar). Similar to PMA treatment, we observed that basal internalization of the LPA₁ receptor required the dileucine motif. The wild-type LPA₁ receptor displayed basal internalization (Fig 25, WT/white bar), whereas the Δ340 and Δ347 mutant LPA₁ receptors did not exhibit any basal internalization (Fig 25, 340,347/white bars). Similar to PMA treatment the Δ353 and Δ361 LPA₁ receptors exhibited basal internalization (Fig 25, 353,361/white bars). These results suggest that the serine rich region is required for LPA-dependent internalization, whereas the dileucine motif is required for basal and PMA-dependent internalization.
Figure 24. Different motifs are required for LPA-induced versus PMA-induced endocytosis

HeLa cells were transfected with the wild-type (WT) and mutant (∆340-∆361) HA tagged- LPA₁ plasmids as described in the materials and methods. The surface receptors were labeled with mouse anti-HA antibody (Covance) at 4°C for 1 hour. The cells were either left at 4°C or transferred to 37°C. The cells at 37°C were then treated with or without either 10μM LPA or 1μM PMA for 30 min at 37°C. After the antibody-receptor complexes were allowed to internalize, surface bound antibodies were removed by mild acid wash and the cells were fixed and processed for immunofluorescence staining as described in materials and methods.
Figure 25. Quantitation of internalization of the mutant LPA₁ receptors
Internalized antibody bound to the LPA₁ receptor was quantified using Simple PCI software as described in materials and methods. Internalization was expressed as the percent of receptor associated antibody following acid wash, relative to the total fluorescence of surface bound antibody of the 4°C control. * p<0.05 – compare Wild-type (WT) to mutants.
9.4 The serine rich region is critical for β-arrestin association

Having established that the serine rich region is critical for signaling desensitization and internalization of the LPA₁ receptor, we next sought to determine if the serine rich region is required for β-arrestin interaction. We had previously shown that in cells expressing the LPA₁ receptor, β-arrestin2 GFP translocates to the plasma membrane upon brief LPA stimulation and that it co-localizes with the LPA₁ receptor (214). HeLa cells were co-transfected with different HA-tagged LPA₁ receptors and β-arrestin2 GFP as shown in Fig 26A. In cells expressing the wild-type LPA₁ receptor, β-arrestin2 GFP translocated to the plasma membrane after 2 minutes of 10µM LPA stimulation (Figure 26A inset, WT/β-arrestin2 GFP), whereas in the cells expressing Δ340 LPA₁ mutant receptor β-arrestin2 GFP failed to translocate to the plasma membrane (inset, 340/β-arrestin2 GFP). As expected, in the cells expressing the Δ347, Δ353 and Δ361 LPA₁ receptor mutants, β-arrestin2 GFP translocated to the plasma membrane (Figure 26A inset - 347, 353, 361/β-arrestin2 GFP). We quantified β-arrestin2 GFP translocation to the plasma membrane in cells after 2 minutes of LPA stimulation (Figure 26B). In the cells expressing the WT LPA₁ receptor, β-arrestin2 GFP translocated to the PM in ~90% of the cells. By contrast, in the cells expressing the Δ340 LPA₁ receptor, β-arrestin2 GFP translocated to the PM in only 10% of the cells. Expectedly, β-arrestin2 GFP translocated to the PM in 70% of the cells expressing the Δ347 LPA₁ receptor. In the cells expressing the Δ353 and Δ361 LPA₁ receptors, β-arrestin2 GFP translocated to the PM in ~90% of the cells. Together these results suggest that the serine rich region is required for β-arrestin interaction with the LPA₁ receptor.
Figure 26. The serine box is required for β-arrestin 2 GFP translocation to the plasma membrane.

a) HeLa cells were co-transfected with the wild-type (WT) or mutant (Δ340-Δ361) HA-LPA1 and β-arrestin2 GFP plasmids as described in the materials and methods. Surface receptors were labeled with mouse anti-HA at 4°C for 1 hour. The cells were washed and then incubated in the absence (data not shown) or presence of 10 µM LPA for 2 min at 37°C. The cells were fixed and processed for immunofluorescence as described in materials and methods. The inset shows a magnified image of the boxed region.

b) Quantitation of β-arrestin2 GFP translocation to the PM after 2 minutes of LPA stimulation. 100 cells were scored for each receptor construct.
9.5 PMA induced internalization of the LPA$_1$ receptor is $\beta$-arrestin independent

The results from Figure 26 and previous studies (214) conclusively showed that upon LPA treatment for 2 minutes, $\beta$-arrestin2 GFP translocates to the plasma membrane. We next sought to determine if PMA treatment also caused $\beta$-arrestin2 GFP to translocate to the plasma membrane. As shown previously, in untreated cells (0 minutes) the LPA$_1$ receptor had a predominantly plasma membrane distribution, whereas $\beta$-arrestin2 GFP had a diffuse cytoplasmic distribution as determined by immunofluorescence. Upon treatment with 10µM LPA for 2 minutes, both the LPA$_1$ receptor and $\beta$-arrestin2 GFP co-localized to punctate structures at the plasma membrane (Figure 27, 2minutes/LPA). Prolonged exposure to LPA for 30 minutes led to an intracellular endosomal distribution of the LPA$_1$ receptor and $\beta$-arrestin2 GFP returned back to the diffuse cytoplasmic staining as in the untreated cells (Figure 27, 30 minutes/LPA). In contrast, upon treatment with 1µM PMA for 2min, $\beta$-arrestin2 GFP did not translocate to the plasma membrane (Figure 27, 2minutes/PMA). These results suggested that $\beta$-arrestin2 GFP does not interact with the receptor upon PMA treatment.

To confirm that the PMA induced internalization of the LPA$_1$ receptor is $\beta$-arrestin independent, we investigated if the LPA$_1$ receptor internalizes in the Mouse Embryo Fibroblasts (MEF’s) derived from $\beta$-arrestin 1/2 double knockout mice. We transfected both MEF’s derived from wild-type mice (MEF WT) and MEF’s derived from $\beta$-arrestin 1/2 KO mice (MEF KO), with the wild-type HA-tagged LPA$_1$ receptor. As shown in Figure 28, in the 4°C untreated MEF WT cells the mouse anti-HA antibody bound to the LPA$_1$ receptor showed a predominantly plasma membrane distribution (Figure 28, MEF WT/ 4°C untreated).
Figure 27. β-arrestin 2 GFP translocation to the plasma membrane is induced by LPA but not PMA treatment.

HeLa cells were co-transfected with the wild-type (WT) HA- LPA₁ and β-arrestin2 GFP plasmids as described in the materials and methods. Surface receptors were labeled with mouse anti-HA at 4°C for 1 hour. The cells were then incubated in the absence (0 min) or presence (for 2 min or 30 min) of either 10 μM LPA or 1 μM PMA at 37°C. The cells were fixed and processed for immunofluorescence as described in materials and methods.
Upon treatment with 10µM LPA or 1µM PMA for 30 minutes, the antibody bound to the LPA$_1$ receptor localized to internal endosomal structures (Figure 28, MEF WT/ 37°C LPA or PMA). By contrast, in the β-arrestin MEF KO cells, upon LPA treatment the antibody bound to the LPA$_1$ receptor failed to internalize (Figure 28, MEF KO/ 37°C LPA), whereas it internalized after PMA treatment (Figure 28, MEF KO/ 37°C PMA). Taken together, these results suggest that the PMA induced internalization of the LPA$_1$ receptor is β-arrestin independent and probably utilizes an alternate adaptor protein.

9.6 AP-2 is required for agonist-independent internalization

The results from Figure 25-28 suggested that PMA-induced internalization of the LPA$_1$ receptor requires a dileucine-based motif and is β-arrestin independent. We next sought to determine the alternate adaptor protein that could be utilized by the LPA$_1$ receptor for agonist-independent internalization. Several studies have shown that tyrosine- and dileucine-based motifs can interact with the adaptor protein AP-2 and mediate internalization (47). We have shown that agonist-independent internalization of the LPA$_1$ receptor does not require β-arrestins but requires a dileucine-based motif (Figure 4 and 5). Therefore, we asked the question if AP-2 was the alternate adaptor protein required for agonist-independent internalization of the LPA$_1$ receptor. We employed the use of siRNA-mediated reduction of AP-2 protein levels to determine if AP-2 is required for agonist-independent internalization. In the untreated cells ~16% of the LPA$_1$ receptor internalized, which represents basal levels of internalization (Figure 29, untreated/siControl). Basal internalization of the LPA$_1$ receptor was inhibited by ~85% after siRNA-mediated knockdown of endogenous AP-2 levels (Figure 29,
untreated/siAP2). Similarly, siAP2 treatment led to a ~86% decrease in PMA-induced internalization of the LPA₁ receptor (Figure 29, PMA/siAP2). Interestingly, LPA-induced internalization of the LPA₁ receptor was partially inhibited by siAP2 treatment leading to a ~50% inhibition (Fig 29, LPA/siAP2). These results were similar to those observed when experiments were performed with Bisindolylmaleimide I (Figure 21). Taken together these results would suggest that agonist-independent internalization of the LPA₁ receptor requires AP-2 and could be regulated by PKC.
Figure 28. PMA induced LPA₁ internalization is β-arrestin independent
MEF WT or MEF β-arrestin 1/2 KO cells were transfected with the wild-type (WT) HA-LPA₁ plasmid as described in the materials and methods. Surface receptors were labeled with mouse anti-HA antibody (Covance) at 4°C for 1hour. The cells were washed and were either left at 4°C or transferred to 37°C. The cells at 37°C were then treated with or without either 10μM LPA or 1μM PMA for 30min at 37°C. After the antibody-receptor complexes were allowed to internalize, surface bound antibodies were removed by mild acid wash and the cells were fixed and processed for immunofluorescence staining as described in materials and methods.
Figure 29. Agonist-independent internalization of the LPA₁ receptor requires AP-2

a) Stably transfected LPA₁/HeLa cells were treated with control siRNA (siControl) or AP-2 siRNA (siAP2) as described in materials and methods. Surface receptors were labeled with mouse anti-HA antibody (Covance) at 4°C for 1 hour. The cells were washed and were either left at 4°C or transferred to 37°C. The cells at 37°C were then incubated in the absence (untreated) or presence of either 10µM LPA or 1µM PMA for 30 minutes at 37°C. After the antibody-receptor complexes were allowed to internalize, the remaining surface bound antibodies were removed by mild acid wash. The cells at both 4°C and 37°C were then fixed and processed for immunofluorescence staining as described in materials and methods. Internalized antibody bound to the LPA₁ receptor was quantified using Simple PCI software as described in materials and methods. Internalization was expressed as a percent of the total fluorescence of surface bound antibody of the 4°C control. * p<0.05

b) Stably transfected LPA₁/HeLa cells were transfected with control siRNA (siControl) or AP-2 siRNA (siAP2) and were stained for AP-2 as described in materials and methods.

c) Cell lysates were prepared from LPA₁/HeLa cells that were transfected with control siRNA (siControl) or AP-2 siRNA (siAP2), separated by SDS-PAGE and were immunoblotted for AP-2 as described in materials and methods.
CHAPTER 10

DISCUSSION

The LPA$_1$ receptor (EDG-2) is the most widely expressed receptor of the Endothelial Differentiation Gene (EDG) family and has been shown to be expressed in a variety of tissues like the heart, colon, brain, and placenta (88, 110). The LPA$_1$ receptor has been shown to be responsible for the migratory potential of multiple cancer cell lines (124, 143, 221, 222). Regulation of receptor expression on the cell surface via trafficking mechanisms, is important in maintaining responsiveness to extracellular signals such as chemotactic attractants (24, 143, 223).

In this study, we examined the role of distinct motifs in the LPA$_1$ receptor tail on its trafficking. Here we show that basal and PMA-induced internalization of the LPA$_1$ receptor is regulated by a dileucine motif in the tail region in an AP-2 dependent manner and is inhibited by a PKC inhibitor, Bisindolylmaleimide I. In contrast, LPA-induced internalization of the LPA$_1$ receptor is regulated by a cluster of serine residues in the tail region, in a β-arrestin-dependent manner.

Several studies have shown a role for PKC phosphorylation in the desensitization and internalization of GPCRs like the purinergic P2Y1, Sphingosine-1-phosphate S1P1 and Gastrin-releasing peptide receptor (15, 224-226). A previous study showed that in rat hepatocytes, a GFP-tagged LPA$_1$ receptor can be phosphorylated by PKC after treatment with phorbol 12-myristate 13-acetate (PMA), leading to its internalization (207). Our results indicate that in LPA$_1$/HeLa cells the LPA$_1$ receptor internalizes upon treatment with PMA (Figure 20B PMA/white bar), which is inhibited by the PKC inhibitor
Bisindolylmaleimide I (Bis I) (Figure 20B PMA/black bar). Additionally, the LPA₁ receptor undergoes basal internalization (Figure 21, untreated), which is also PKC-dependent as it is sensitive to Bis I treatment. Upon treatment with LPA, ~30% of the surface LPA₁ receptor internalizes into endosomes (Figure 21, LPA) but pre-treatment with Bis I, prior to LPA stimulation, did not completely inhibit internalization but reduced it to about ~20% as compared to ~30% in the control. Upon LPA stimulation, the LPA₁ receptor can activate Goq and phospholipase C and subsequently activate PKC thus raising the possibility that PKC has a feedback inhibitory mechanism. Additionally, certain studies have shown that PKC inhibitors can inhibit the activity of adaptor associated kinase-1 (AAK1), which phosphorylates the μ-subunit of AP-2, inhibiting internalization (227).

We have previously shown that LPA-dependent internalization of the LPA₁ receptor is β-arrestin and clathrin-dependent (214). Previous studies have suggested that agonist-independent internalization of certain GPCRs do not require β-arrestin but utilize an alternative adaptor protein, AP-2, which can target receptors to Clathrin Coated Pits (CCPs) for internalization (33). Our studies suggest that PMA-induced internalization of the LPA₁ receptor does not require β-arrestin since PMA readily induced internalization of LPA₁ receptors in the β-arrestin 1/2 KO MEF cells (Figure 28). Unlike LPA stimulation, we observed that PMA treatment did not induce β-arrestin translocation to the plasma membrane (Figure 27). These results collectively suggest that PMA-induced internalization is β-arrestin independent.

β-arrestin dependent internalization has been shown to require clusters of serine residues on GPCR tails for their association (51, 65). On analyzing the LPA₁ receptor tail
we found a serine rich region between residues 340 and 347. Our studies indicate that indeed, the serine rich region (340-347) is required for β-arrestin translocation to the plasma membrane upon brief LPA stimulation (Figure 26). It is known that β-arrestins are required for both signal desensitization as well as endocytosis of GPCRs (17, 228). Indeed, the serine rich region (340-347), as expected, was also required for LPA-dependent internalization of the LPA₁ receptor (Figure 25, LPA). Just as in endocytosis, GPCRs that are faulty in their β-arrestin binding abilities, fail to desensitize. As expected, the serine rich region is critical for signal desensitization as determined by the observation that the mutant LPA₁ receptor lacking the serine rich region (Δ340) has elevated levels of phosphoinositide hydrolysis as compared to those receptors that contain the serine rich region (Figure 23). Thus LPA₁, which is a Class A GPCR as defined by Oakley et. al (31), requires a cluster of serine residues for LPA-dependent transient β-arrestin association and subsequent internalization.

We also observed that the LPA₁ receptor tail has two dileucine motifs, one proximal and one distal to the trans-membrane domain. The proximal dileucine motif precedes a dicysteine motif, which is a potential palmitoylation site. Previous studies have shown that dileucine motifs preceding dicysteine motifs are important in regulating ER to Golgi transport (48, 229). Hence, we decided to pursue the distal dileucine motif for our studies. Dileucine motifs have also been shown to be critical in PKC-dependent endocytosis of GABA receptors (61, 230). We had observed in Figure 21 that both constitutive and PMA-induced internalization are PKC-dependent. Studies with truncation mutant LPA₁ receptors showed that upon PMA stimulation, mutant LPA₁ receptors that lack the dileucine motif (Δ340, Δ347) fail to internalize, whereas mutant
LPA_1 receptors that have the dileucine motif internalize like wild-type LPA_1 receptors (Figure 25). In contrast, the dileucine motif does not seem to be required for LPA-dependent internalization. As in the case of PMA stimulation, basal internalization also seems to depend on the dileucine motif for internalization (Figure 25, Untreated). Taken together these results confirm the observations in Figure 21 and suggest that both basal and PMA-induced internalization follow a similar PKC- and dileucine motif-dependent pathway. Studies with another EDG family GPCR, S1P1 (EDG-1), have shown that this receptor utilizes two distinct mechanisms, GRK-dependent and PKC-dependent mechanisms, to regulate agonist-dependent and agonist-independent internalization, respectively (15). Our studies show that the LPA_1 (EDG-2) receptor also utilizes two distinct mechanisms for agonist-dependent versus agonist-independent internalization. Lysophospholipid receptors (LPA and S1P) have been shown to induce diverse cellular responses such as proliferation, migration, differentiation and survival (116). Certain types of ovarian cancer over-express the LPA2 receptor but not the LPA_1 receptor (231). In fact, restoring expression of LPA_1 in ovarian cancer cells causes apoptosis and anoikis (139). Additionally, a study showed that the LPA_2 receptor is a more potent activator of G_αq as compared to the LPA_1 receptor (180). It would be of great interest to determine if the LPA_2 receptor causes heterologous desensitization of the LPA_1 receptor in these cancer cells and the role of the LPA_1 dileucine-based motif in this context.

Previous studies have shown that GPCRs that internalize in an agonist-independent manner can utilize AP-2 as an adaptor protein and that tyrosine- and dileucine-based motifs on these receptors can interact with AP-2, mediating
internalization (47, 54, 232). Therefore we tested to see if AP-2 is required for agonist-independent internalization of the LPA₁ receptor. Treatment with siRNA to reduce AP-2 expression levels resulted in a ~ 90% decrease in both basal and PMA-induced internalization (Figure 28, untreated, PMA/siAP2), suggesting that both PMA-induced and basal internalization utilize a similar AP-2 dependent pathway of internalization. LPA-induced internalization was also partially inhibited by siAP-2 treatment (Figure 28, LPA/siAP2). This partial inhibition is due to the fact that siRNA-mediated reduction of AP-2 has been shown to cause a 10-fold decrease in the number of clathrin-coated pits at the plasma membrane (185). Therefore, these results suggest that AP-2 is not necessary for LPA-induced internalization and utilizes an alternate adaptor protein, β-arrestin, for internalization.

GPCRs like the LPA₁, CXCR4, and PAR1 are constitutively internalized and either have tyrosine- or dileucine-based motifs on their tails and confer a migratory potential to cells that express them. Is there a correlation between tyrosine- or dileucine-based motifs, constitutive internalization, and cell migration? Do other GPCRs that constitutively internalize and have tyrosine- or dileucine-based motifs, confer migratory potential to cells that express them? If indeed the hypothesis that regulation of receptor expression and turnover by basal internalization can affect cell migration towards a chemo-attractant like LPA is true, then the cells in which receptors fail to basally internalize (Δ340, Δ347 – LPA₁) should fail to migrate. It would be of great physiological importance if indeed basal internalization is a mechanism that regulates cell migration. Further studies need to be done to determine the role of basal trafficking of receptors and their effect on cell migration in different contexts.
CHAPTER 11

FUTURE DIRECTIONS

Having deduced the molecular determinants for agonist-independent internalization, the next question that arises is what is the physiological relevance of agonist-independent internalization? Several studies have suggested that constitutive trafficking may play a role in the differential spatial expression of GPCRs. Studies with the UNC5A receptor in neurons have shown that constitutive trafficking is required for targeting the receptor to the axonal regions and inhibition of constitutive trafficking leads to mislocalization of the receptor to the cell body. Thus constitutive trafficking of the LPA1 receptor could also play a role in its spatial localization on the plasma membrane and thereby contribute to perceiving extracellular signals that are also spatially oriented. This might not be relevant to a cell culture system but very relevant to an in vivo system, as cells in vivo are generally polarized or have different spatial orientations relative to their external environment. It is known that the LPA1 receptor confers migratory potential to cells that express it. Further studies need to be done to elucidate the role of the LPA1 dileucine motif and AP-2 in cell migration towards a chemoattractant. Based on the studies with PMA-induced internalization of the LPA1 receptor it has been suggested that this LPA1 internalization can be a result of heterologous desensitization. Thus studies need to be done where the LPA1 receptor is co-expressed with another Gαq-coupled receptor to determine if activation of one receptor leads to the internalization of the other.
APPENDIX A

PROTOCOLS

Splitting Mammalian Cells

1. Rinse cells with 1 mL trypsin-EDTA.
2. Add 2 mL trypsin and incubate for 1-2 minutes to loosen cells.
3. Add 3 mL complete media to cells and pipet cells 10 times to get uniform suspension.
4. Place 10 mL fresh, warm media into a new 10 cm dish.
5. Add 0.4-0.6 mL suspended cells to new flask to get a 1:10 dilution.

Plating Mammalian Cells

1. Place several acid washed coverslips into a 10 cm petri dish.
2. Pour 70% EtOH over the coverslips.
3. Flame 4-5 slips at a time very quickly and then let air dry for a few seconds. (Make sure that all EtOH is evaporated or cells will not grow on the slip.)
4. Place ~20 slips in a sterile tissue petri dish for plating.
5. Place 10 mL of complete media into the petri dish.
6. Add a few drops of suspended cells (from flask spilt) to the dish.
7. Add appropriate drug/inhibitor and place in CO2 incubator overnight to do transfection next afternoon. If cells are plated early in morning, then they can be transfected that same evening.

Lipofectamine Transfection

For a transfection in a 24-well dish:
1. Plate 40,000 HeLa cells in complete media into each well for ~24 h.
2. The following day, change the media to OptiMEM serum free medium in each well.
3. For each transfected row (6 wells total/row) dilute a total of 1.0 µg/well of DNA into 300µl serum-free media into one labeled tube. Repeat for each row.
4. Incubate the mixture for 5 min.
5. Dilute lipofectamine into another labeled tube at the ratio (0.4µg DNA: 1µl lipofectamine) with 300 µl serum-free media.
6. Immediately combine the tube with DNA and the tube with lipofectamine and incubate the mixture for 20-45 min.
7. Add 100 µl of the mixture into each well of the 24-well plate.
**Lipofectin Transfection**

For a Transfection in a 6 Well Dish:
1. Plate cells to get recommended cell density of 40-60% confluent on day of transfection.
2. For each transfection:
   a. dilute 1 µg of DNA into 100 µl OptiMEM media into 1 labeled tube
   b. dilute 2 µl (per 1 µg plasmid) Lipofectin into 100 µl of OptiMEM media into 1 labeled tube
3. Let stand at room temperature for 30 – 45 min.
4. Combine the two solutions and let stand for 15 min at room temperature.
5. Wash cells in 6 well once with 2 mL OptiMEM and replace with 1.8 mL OptiMEM.
6. Add DNA/Lipofectin mix to the well and place in 37°C incubator for 4-6 h.
7. Replace the DNA containing media with DMEM media – antibiotics and return to incubator for 48-72 h.

**ExGen 500 in vitro Transfection**

Considerations:
1. High quality DNA of 1.8 OD ratio or higher is recommended.
2. Recommended cell density is around 50% at time of transfection.
4. Transfection efficiency is higher in the presence of serum w/o antibiotics.

Day 1: Seed 0.45x 10⁶ HeLa cells (density depends on cell type) in a 10 cm dish containing complete DMEM (-antibiotics).
Day 2: Transfect in the morning. Prior to transfection, transfer coverslips to a 6-well plate containing 2 mL of complete DMEM (-antibiotics).
Day 3: Change the media.
Day 4: Perform the assay.
Day 2: Procedure for 6-well plate: Use 1 µg :3.3 µl ratio of DNA to Ex-Gen.
   1. Dilute recommended amount of DNA (Total 2.0 µg/6-well) into 200 µl of 150 mM NaCl.
   2. Vortex briefly and spin down.
   3. Add 7.0 µl of ExGen500 to DNA solution and immediately vortex for 10 sec.
   4. Incubate at room temperature for 10 min.
   5. Add the ExGen500/DNA mixture to one well of 6-well plate and place on shaker for 5 min.
   6. Incubate for 24 h and change media following day.
   7. Assay 48 h after transfection.

**Indirect Immunofluorescence**

1. Day 1: Grow cells on 12 mm acid-washed No. 1 circle glass coverslips in a 10 cm dish. Coverslips should sit in 70% ETOH. Flame coverslips prior to use and transfer to 10cm dish. Density of cells depends on cell type. (HeLa – 0.45x10⁶ ; MEFs – 1.0x10⁶)
2. Day 2: Transfer individual coverslips to the wells of a 24, 12, or 6-well dish that contains the appropriate amount of media. Begin transfection protocol.
3. Day 3: Remove media and replace with serum free or complete media.
4. Day 4: Treat as required for experimental protocol.
5. Transfer coverslip to one well in a 12-well dish containing 1 mL of 2% formaldehyde in PBS pH 7.4.
   a. 2% formaldehyde in PBS
      i. Add 27 mL of 37% formaldehyde stock into a graduated cylinder.
      ii. Fill to 500 mL with PBS pH 7.4
6. Incubate for 10 min at room temperature (RT).
7. Remove fixative and add 1 mL of 10% Adult calf serum in PBS (PBS/serum/azide) and incubate for 5 min at room temperature. This can be stored overnight at 4°C.
   a. PBS/serum
      i. Add 50 mL of calf serum to 500 mL graduated cylinder.
      ii. Add 0.5 mL of 20% sodium azide stock soln.
      iii. Fill to 500 mL with PBS pH 7.4
8. Dilute primary antibodies into PBS/serum containing 0.2% saponin and spin for 5 min at 14,000 rpm.
   a. PBS/serum + 0.2% saponin
      i. Add 20 µl of 10% saponin stock (made in dH₂O) to microfuge tube
      ii. Add 980 µl of PBS/serum for a total of 1 mL
9. Place a piece of parafilm in the bottom of a 150 mm petri dish and label with numbers corresponding to 12-well dish.
10. Add 25 µl of the appropriate diluted antibody solution to each spot on the parafilm.
11. Pick up individual coverslips with tweezers and wick excess fluid on paper towel.
12. Invert coverslip onto antibody drop (i.e. cell side down), cover dish and incubate in a bench drawer for 1 h.
13. Carefully transfer coverslip, cell-side up, back into 12-well dish.
14. Wash coverslips with 1 ml PBS/serum (3 x for 5 min).
15. Dilute fluorescently-labeled secondary antibodies in PBS/serum + 0.2% saponin and spin for 5 min at 14,000 rpm.
16. Invert coverslips onto 25 µl drops of antibody on parafilm as described above and incubate for 1 h.
17. Wash coverslips 3 x 5 min with PBS/serum.
18. Rinse coverslips with PBS alone and mount onto glass slides with fluoromount G and seal with nail polish.

**Metamorph Co-localization**

1. Open and load image of interest
2. Deconvolute images prior to quantitation
3. Process Menu
4. Select “2D deconvolution”
5. Click nearest neighbor and Apply (adjust if needed)
Display color combine
3. Display Menu
Select “Color Separate”
Click red, green or blue ---“new”
4. Select rectangle box in Regions tools and place in Blank region of image
5. Regions Menu
Select “Transfer Region” to place blank in all colors of image
6. Measure Menu
Select “Show Region Statistics”
*the “Use Threshold” box should NOT be checked
*region around box should be blinking (active)
*Add the sum of average and standard deviation computed for each color image. Record measurements for each color.
7. Measure Menu
Select “Threshold Image”
*Make sure State is Off
Insert the values derived from previous step into the “Low Intensity” box
8. Region Tools
Select line or box tool to mark the areas of image to analyze. Double click to activate.
9. Regions Menu
With image outline blinking, select Transfer Region
Transfer outline of interest to all the color images separated earlier
10. Measure Menu
Select “Show Region Statistics”
*Check the “inclusive” box for each color of the image
* Add the sum of the average and standard deviation computed
11. Measure Menu
Select “Threshold Image”
*input the sum calculated above into the “Low intensity” box
12. Applications Menu
Select “Measure colocalization”
*set image to “A” or “B” as appropriate
*check the “show percentage box”
*log into Excel spreadsheet

Loss of Surface receptor (LOSR) Quantitation Assay

1. Grow cells directly on bottom of wells that contain 1 mL media. (this works best at 150,000 cells per well).
2. Treat as required for experimental protocol.
3. Remove medium and fix cells with 1 mL of 2% formaldehyde in PBS pH 7.4.
   2% formaldehyde in PBS
   i. Add 27 mL of 37% formaldehyde stock into a graduated cylinder.
   ii. Fill to 500 mL with PBS pH 7.4
4. Incubate for 10 min at room temperature (RT).
5. Remove fix and add 1 ml of 10% Adult calf serum in PBS (PBS/serum) and incubate for 5 min at room temperature. This can be stored overnight at 4°C.
   - **PBS/serum**
     - i. Add 50 mL of calf serum to 500 mL graduated cylinder.
     - ii. Fill to 500 mL with PBS pH 7.4
6. Dilute primary antibodies into PBS/serum without saponin and spin for 4 min at 14,000 rpm.
   - b. Mouse anti Flag use at 1:750. (stock = 2-5 mg/ mL)
   - c. ConA Biotin use at 1:750. (stock = 5 mg/ mL) (used at 5 µg/ mL) (Vector Laboratories, B-1005)
7. Remove the PBS serum from the wells.
8. Add 250 µl of primary antibody to each well.
9. Cover dish and incubate in drawer for 45 min.
10. Wash cells with 1 ml PBS/Serum (3 x 3 min).
11. Dilute fluorescently-labeled secondary antibodies in PBS/serum without saponin and spin for 4 min at 14,000 rpm.
   - d. Goat anti Mouse HRP Conjugate use at 1:1000. (stock = 0.8 mg/mL) (used at 0.3 µg/mL) (Promega, W4021)
   - e. AP Streptavidin use at 1:1000. (stock = 1 mg/mL) (used at 5 µg/mL) (Alkaline Phosphatase Streptavidin, SA-5100, Vector Laboratories)
12. Remove the PBS Serum and add 250 µl secondary antibody to each well.
13. Cover and incubate in bench drawer for 45 min.
14. Wash cells with 1 mL PBS/Serum (3 x 3 min).
15. Aliquot out approximate amount of 1 Step ABTS necessary and warm it up in dH₂O bath, keeping stock bottle in fridge. (Pierce, 37615)
16. Rinse cells with 1 mL 1 X PBS alone for 5 min.
17. Aliquot out approximate amount of pNPP/Sodium Bicarbonate
   - pNitroPhenylPhosphate (pNPP) (vector Laboratories, SK5900)
   - add 5 drops per 2 mL of 100 mM Sodium Bicarbonate
   - 100 mM Sodium Bicarbonate
   - 75 ml dH₂O
   - 0.84 g Sodium Bicarbonate
   - pH to 10.0 with NaOH
   - fill to 100 mL with dH₂O
18. Remove PBS.
   - f. Add 150 µl pre-warmed ABTS to wells stained with Donkey anti Mouse HRP and to first column of a 96 well dish to be used as a standard.
   - g. Add 150 µl pNPP solution to wells stained with AP Streptavidin and to first column of a second 96 well dish to be used as a standard.
   - h. Incubate on benchtop for 15 min.
19. After the 15 min, add 150 µl 1% SDS to all wells containing ABTS, including those of the 96 well dish.
20. a. Mix thoroughly by pipetting up and down and quickly add all 300 µl of
ABTS/SDS wells to corresponding well of 96 well dish.
  b. Mix thoroughly by pipetting up and down and quickly add all 150 µl of pNPP
  wells to corresponding well of 96 well dish.
  c. ABTS/SDS wells should turn green
  d. pNPP wells should turn yellow

21. Read absorbances on plate reader at 405 nm in SoftMax Pro Program.
  e. Assay for ABTS/SDS: Endpoint ELISA: HRP and ABTS w SDS stop
  f. Assay for pNPP: Endpoint ELISA: AP and pNPP

**BCA Protein Concentration Assay**

1. Make BSA standard (1 mg/mL) solutions by adding the appropriate amount of BSA to
   microfuge tubes (0 µl, 5, 10, 15, 20, 25).
2. Add 5 µl of protein sample to a new microfuge tube.
3. Add 0.5 ml of BCA mixture to each microfuge tube:
   1 part Soln. B to 50 parts Soln. A
4. Incubate for 30 min at 37° C.
5. Read at 562 nm on plate reader.

**SDS-PAGE Gel Recipes**

*MINIGEL: SEPARATING GEL (10ml)*

**Reagent 8% 10% 13% 15%**

40% Acrylamide 2ml 2.5ml 3.25ml 3.75ml
1.5M Tris, 0.4% SDS pH 8.8 2.5ml 2.5ml 2.5ml 2.5ml
ddH₂O 5.5ml 5ml 4.25ml 3.75ml
10% APS 50µl 50µl 50µl 50µl
TEMED 10µl 10µl 10µl 10µl

*MINIGEL: STACKING GEL (10ml)*

40% Acrylamide .75ml
0.5M Tris, 0.4% SDS pH 6.8 2.5ml
ddH₂O 6.75ml
10% APS 100µl
TEMED 12µl

**SDS-PAGE Set Up**

1. Insert comb into rig and mark a line about 1 cm below the bottom of comb.
2. Add appropriate percentage separating gel first using a Pasteur pipet. This can store at 4°C overnight. Leave pipet in gel mixture and wait to harden (about 10-15 min).
3. Add a layer of 0.1% SDS.
4. Pour stacking gel using a Pasteur pipet taking care to get rid of bubbles. Insert comb into rig, making sure no bubbles form and allow this to dry like the separating gel.
5. Load gels into running rig, short plate towards the inside.
6. Add 1X SDS running buffer in between 2 gel rigs, check for leaks.
7. Load bench mark standard and samples with 2X or 4X sample buffer.
8. Fill 1X SDS running buffer on out side of gel rig till it covers the wire running across the inside of gel rig.
9. Run gel at ~150V until the dye runs out into the running buffer, usually 1 h.

**Cell Lysis (Western Blotting)**

1. Rinse culture dishes of cells twice with ice-cold 1X PBS
2. Scrape cells into a pool at the bottom of the dish using 1X PBS with protease and phosphatase inhibitors added fresh each time
3. Transfer pool of cells into an ice-cold microfuge tube and spin in a cold centrifuge at 500-1200 rpm for about 5-15 min.
4. Add 50 µl (A549) or 200-500 µl (HeLa, HepG2) of lysis buffer to the cell pellet after removing the supernatant.
   **Lysis Buffer:**
   - 1% NP-40 1mL
   - 1% deoxycholate salt 1g
   - 0.15M NaCl (from 5M stock) 3mL
   - 0.1% SDS (from 20% stock) 0.5mL
   - 0.01M sodium phosphate 7.2 10mL
   - 2mM EDTA (from 0.5M stock) 400µl
   - 50mM NaF (from 1M stock) 5mL
   - 0.2M orthovanadate (from 0.1M stock) 2mL
   - H₂O (to 100mL total volume) 78mL
   *Fresh protease inhibitors each time
5. Allow the cells to lyse on ice for 30 min with vortexing every 10 min.
6. Spin in the cold centrifuge for 15 min, remove supernatant for BCA Assay.

**Western Blotting (Chemiluminiscence Detection)**

1. After electrophoresis, soak gel in transfer buffer (chilled) for 5-10 min.
2. Assemble sandwich in the order indicated on the black side of cassette:
   a. Scotch-brite pad
   b. Whatman filter paper (cut slightly larger than the gel)
   c. SDS-PAGE Gel
   d. Nitrocellulose paper (roll out bubbles with pipet)
   e. Whatman filter paper (roll out bubbles with pipet)
   f. Scotch-brite pad
3. Seal cassette, place in apparatus, fill with transfer buffer, add a stir bar, and place the ice pack in the back.
4. Place on stir plate and stir, hook up leads (- to – and + to +)
5. Transfer at 100V for 1 h.
7. Block nitrocellulose filter with 5% milk/0.1% Tx-100/PBS for 1 h on shaker.
8. Remove blocking solution.
9. Incubate with 1° Ab diluted in milk solution for 1 h on shaker.
10. Wash with Triton/PBS (1x 15 min, 3x 5 min).
11. Incubate with 2° Ab for 1 h on shaker (HRP conjugated Donkey 1:5000).
12. Wash with Triton/PBS (1x 15 min, 4x 5 min).
13. Treat on saran wrap with ECL soln (1:1 mixture of Soln A and Soln B; make up just before use) 1 min, and expose the film (start with 1 min exposure).

**MAP Kinase Antibody Western Blot**

1. Block nitrocellulose with TBST/5% milk for 30 minutes at room temperature.
2. Decant the milk and wash with TBST 3 times.
3. Cover with TBST/0.1% BSA containing anti-phospho or anti-non phospho MAPK antibody (1:1000) at 4°C overnight.
4. Decant antibody solution.
5. Wash 5 times with 20 ml of TBS-Tween for 5 min each.
6. Apply TBS-Tween/5% milk containing Goat anti-Rabbit HRP (1:1000) for 1 h at room temperature with agitation.
7. Wash 5 times with 20 ml of TBS-Tween for 5 min each.
8. Soak for 1 min in West Pico Chemiluminiscent Reagent.

**Immunoprecipitation Assay**

1. Transfect HeLa cells in 35 mm dishes.
2. Rinse cells twice with ice-cold PBS, add 0.5 mL of lysis buffer and incubate on ice 10 min.
3. Scrape cells and collect lysate in microfuge tubes, vortex to break up clumps.
4. Spin in the chilled centrifuge at 14,000 rpm (or top speed), at 4°C for 15 min.
5. Transfer lysate to fresh tube, assay 10 µl for protein concentration with BCA mix. (BSA stds: 0, 2, 4, 6, 8, 10, 15, and 20 µg)
6. Wash 30 µl protein A/G-agarose beads with PBS.
7. Transfer equal amount of lysate to tube with protein A/G-agarose beads and add 1 µl of mouse anti-HA antibody (Covance). Tumble for 2 h at 4°C.
8. Pellet beads and wash 3x with IP wash buffer.
9. Add 30µl of 2X reducing sample buffer, boil 5 min.
10. Pellet beads and load into a 10% SDS gel and proceed with western blotting.
Making Stable Cell Lines

1. Transfect cells with appropriate DNA.
2. On day supposed to do experiment, change media and let cells grow another day.
3. Next day trypsinize cells and split 1:25 into 5, 10 cm dishes. Cover with 10 mL complete media containing 0.05 mg/mL G418.
4. For 1 week, feed and treat cells with G418 (100 µl of the solution per dish).
5. When colonies are large enough to see, pick them.
   - place 1 mL of complete media into 12 well dishes with 0.05 mg/mL G418
   - add 25 µl trypsin to lid of each well.
   - mark 5 well isolated colonies on each dish
   - remove media from cells
   - pick colony with P100 tip and transfer onto drop of trypsin with ~5 µl of trypsin in tip
   - let sit for 2-5 minutes
6. Add cell/trypsin mixture to corresponding well of 12 well dish.
7. Place into CO2 incubator.
8. Feed cells every 3 – 4 days.
9. When definite colonies appear in 12 wells split into 6 well dishes with one coverslip. Test the coverslip by Immunofluorescence for stable transfection.
10. When stably transfected cell line has been identified, freeze it down.

Simple PCI quantitation

1. Open new Workfile document
   
   File> New>Workfile document

2. Set imaging parameters
   - Click capture icon (top left corner)
   - Set exposure time and gain
   - Capture image
   - Click OK (this will save settings to workfile)

3. Set quantitation parameters
   - Use icons going down on the left side of the screen
   - Use only identify and measure
     - Identify (set threshold min and max, click ok)
     - Measure (select measurements - area, greylevel and total grey)
   - All of these changes will be saved to the workfile document.

4. Collect data
   - select start collecting button (trafficlight icon)
   - Create data file box appears – choose save location
   - select start icon (arrow)
   - Capture window will open
- capture image
- click ok
- Software will automatically identify objects, quantify and return to the capture screen
- When finished collecting images for a data set, click stop collecting button in capture window

5. Analyze data
- Data will be stored in columns as area, greylevel and total grey.
- Total grey corresponds to total fluorescence in the captured picture
- convert data file to excel and analyze data.
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