Characterisation of Human Airway Smooth Muscle Cell Lysophosphatidic Acid Receptors in Asthma and Health

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Abstract

Characterisation of Human Airway Smooth Muscle Cell Lysophosphatidic Acid Receptors in Asthma and Health – Marie Valente

Lysophosphatidic acid (LPA) is a major serum phospholipid and bioactive lipid mediator that exerts a broad range of effects through its family of cognate G protein-coupled receptors via coupling to multiple G proteins and can affect a variety of cellular functions in a range of tissues and cell-types. LPA is known to affect airway function and a growing body of evidence suggests a role for LPA receptor function in the molecular aetiology of asthma.

The aims of this project were to characterise the function of LPA and its receptors in cultured human airway smooth muscle (hASM) cells from asthmatic and control donors. Expression of a broad range of genes related to LPA metabolism/signalling and inflammatory signalling were analysed using both traditional and high throughput RT-qPCR methods. Decreased expression of the genes for a phosphodiesterase, PDE4B, and an LPA metabolising enzyme, LPP2, was detected in hASM cells derived from asthmatic patients compared to control donors. Investigation of LPA receptor pharmacology using a $[^{35}\text{S}]$GTPγS binding assay in a model cell-line showed that LPA species with a range of fatty acid tail lengths and degrees of saturation were active at LPA receptors with varying potencies. LPA exhibited complex modulation of forskolin and isoprenaline induced cAMP responses in hASM cells. Though no differences in the effects of LPA treatment were observed in the two disease-states studied, hASM cells from asthmatics exhibited a significantly higher magnitude of cAMP response compared with those from control donors, which could indicate dysfunctional adenylyl cyclase or PDE activity in disease.

These findings confirm that LPA is able to affect hASM cell function in ways pertinent to asthma's pathophysiology, but do not indicate altered LPA receptor expression or function in disease.
Acknowledgements

Firstly, I would like to thank my PhD supervisor, Prof. John Challiss, for his help and guidance throughout the project and particularly for his patience and encouragement during the writing stage. I would also like to thank my industrial supervisor, Dr. Mark Dowling, for his advice and support during my stints at Novartis and to Prof. Chris Brightling for his advice on clinical aspects and for kindly allowing me use of the human samples. Thank you to the BBSRC and Novartis for supporting this project financially.

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ABSTRACT ................................................................. I

ACKNOWLEDGEMENTS .................................................... II

CONTENTS .................................................................. III

ABBREVIATIONS ........................................................... VI

CHAPTER 1. INTRODUCTION ............................................. 1

1.1. LYSOPHOSPHATIDIC ACID .............................................. 2
  1.1.1. Lyso phosphatidic acids and their structure ......................... 2
  1.1.2. LPA-binding proteins ............................................... 2
  1.1.3. LPA metabolism .................................................. 4
1.2. G PROTEIN-COUPLED RECEPTORS .................................. 5
  1.2.1. The G protein-coupled receptor (GPCR) superfamily .......... 5
  1.2.2. Guanine nucleotide-binding (G) proteins ......................... 7
  1.2.3. G protein-mediated signalling ..................................... 8
  1.2.4. G protein-independent signalling .................................. 10
  1.2.5. Tools for studying GPCR-G protein-dependent signalling .......... 12
1.3. LPA RECEPTORS .......................................................... 14
  1.3.1. The lysophosphatidic acid receptor family .......................... 14
  1.3.2. The LPA receptor-autotaxin axis .................................... 22
  1.3.3. Physiological and pathophysiological roles for LPA and its receptors ........................................ 24
1.4. LPA AND ITS RECEPTORS IN RESPIRATORY PHYSIOLOGY AND DISEASE ........................................ 25
  1.4.1. Asthma .................................................................... 25
  1.4.2. Production of LPA and expression of LPA receptors in the airways ........................................ 26
  1.4.3. LPA and its receptors in airway inflammation ......................... 27
  1.4.4. LPA and its receptors in airway remodelling .......................... 30
  1.4.5. Effects of LPA and its receptors on contractility ...................... 31
  1.4.6. LPA and its receptors in models of asthma ............................ 32
  1.4.7. Role of LPA and its receptors in pulmonary fibrosis ................. 33
1.5. PROJECT AIMS ............................................................ 33

CHAPTER 2. MATERIALS AND METHODS ................................ 35

2.1. MATERIALS ................................................................ 36
2.2. CELL CULTURE METHODS ............................................ 36
  2.2.1. Culture of cell lines ................................................ 36
  2.2.2. Isolation, establishment and culture of primary human airway smooth muscle cells ................ 39
CHAPTER 4. PHARMACOLOGICAL PROFILING OF LPA RECEPTOR SUBTYPES ........................................ 91
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
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<tr>
<td>AR</td>
<td>Adrenoceptor</td>
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<td>ASM</td>
<td>Airway smooth muscle</td>
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<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ATX</td>
<td>Autotaxin</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>c.p.m.</td>
<td>Counts per minute</td>
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<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca$^{2+}$/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDS</td>
<td>Coding DNA sequence</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disorder</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DDP</td>
<td>Dodecyl fatty acid phosphate</td>
</tr>
<tr>
<td>DGPP</td>
<td>Diacylglycerol pyrophosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>Edg</td>
<td>Endothelial differentiation gene</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>EGTA</td>
<td>Ethyleneglycoltetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAF-BSA</td>
<td>Fatty acid-free bovine serum albumin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDSS</td>
<td>Functional drug screening system</td>
</tr>
<tr>
<td>FK</td>
<td>Forskolin</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl pyrophosphate</td>
</tr>
<tr>
<td>Fura-2-AM</td>
<td>Fura-2-acetoxymethylester</td>
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<tr>
<td>G protein</td>
<td>Guanine nucleotide-binding protein</td>
</tr>
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<td>G418</td>
<td>Geneticin sulphate</td>
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<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5’-diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GINA</td>
<td>Global initiative for asthma</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein-coupled receptor kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>Guanosine 5’-O-[γ-thio]triphosphate</td>
</tr>
<tr>
<td>hASM</td>
<td>Human airway smooth muscle</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>Iso</td>
<td>Isoprenaline</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KHB</td>
<td>Krebs-Henseleit buffer</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin-cholesterol acyltransferase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>LPE</td>
<td>Lysophosphatidylethanolamine</td>
</tr>
<tr>
<td>LPP</td>
<td>Lipid phosphate phosphatase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lysophosphatidylerine</td>
</tr>
<tr>
<td>LysoPLD</td>
<td>Lysophospholipase D</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>NAG</td>
<td>N-Arachidon酰glycine</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor κ-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-specific binding</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>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Ca²⁺-dependent protein kinase</td>
</tr>
<tr>
<td>PLA</td>
<td>Phospholipase A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PTx</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulator of G protein signalling</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-dependent kinase</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Suppressor of cytokine signalling 1</td>
</tr>
<tr>
<td>SPA</td>
<td>Scintillation proximity assay</td>
</tr>
<tr>
<td>SRE</td>
<td>Serum response element</td>
</tr>
<tr>
<td>ST2</td>
<td>Suppression of tumorigenicity 2</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline-Tween</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TLDA</td>
<td>TaqMan® low density array</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UDG</td>
<td>Uracil-DNA glycosylase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Y-27632</td>
<td>Trans-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride</td>
</tr>
</tbody>
</table>
CHAPTER 1. Introduction
1.1. **Lysophosphatidic Acid**

1.1.1. **Lysophosphatidic acids and their structure**

Lysophosphatidic acids (LPAs) are simple glycerophospholipids that comprise a phosphate, a glycerol and a single fatty acid group (Figure 1.1A). The glycerol backbone can be acylated at either the sn-1 or sn-2 positions by any of a variety of fatty acid acyl groups. LPA species, including short and long chain (e.g. 6:0, 16:0, 18:0) with varying degrees of unsaturation (e.g. 16:1, 18:1, 20:4), have been detected in a range of biological fluids, including saliva (Sugiura et al., 2002), bronchoalveolar lavage (BAL) fluid (Georas et al., 2007), plasma, and at micromolar concentrations in serum (Baker et al., 2001). Of these fluids, serum is by far the best characterised and platelets are thought to be the main source of this LPA and its precursors (Aoki et al., 2002). Interestingly, LPA levels are increased in a number of pathological conditions including lung injury (Tager et al., 2008), allergen-challenged asthmatics (Georas et al., 2007), hepatitis C (Watanabe et al., 2007) and cancer (Sedlakova et al., 2008).

1.1.2. **LPA-binding proteins**

In the physiological environment LPA is thought to exist bound to a variety of protein carriers (van der Bend et al., 1992) including albumin (Tigyi and Miledi, 1992) and it has been proposed that binding proteins for LPA contribute to its activity at receptors (Hama et al., 2002). Albumin is well established as a plasma antioxidant and fatty acid-binding protein. LPA binds to the high affinity fatty acid-binding sites of albumin with greater affinity than the structurally similar lysophospholipids, lysophosphatidylcholine or lysophosphatidylethanolamine (Thumser et al., 1994). LPA bound to albumin has been shown to be the factor responsible for stimulation of oscillatory currents in *X. laevis* oocytes and neurite retraction of PC12 cells in the presence of serum albumin (Tigyi et al., 1991; Tigyi and Miledi, 1992). Binding to albumin helps prevent degradation of LPA by lipases (Tigyi et al., 1991) so it is likely that albumin protects LPA from biological breakdown *in vivo*.

LPA binds to the plasma form of the actin binding protein, gelsolin, at the same site that it binds phosphatidylinositol-4,5-bisphosphate (PIP$_2$; Mintzer et al., 2006). LPA binds gelsolin with a higher affinity than albumin (Goetzl et al., 2000). Gelsolin exists in two
**Figure 1.1**

A, structure of LPA, highlighting its phosphate, glycerol and fatty acid acyl groups and illustrating the position of the sn1 and sn2 carbons. Here, LPA (18:1) is shown. B, diagram showing the proposed routes for LPA production.
forms: cytoplasmic and the secreted plasma form. Within the cell gelsolin can sever actin filaments and cap their barbed, growing ends so is thought to play a role in the regulation of cytoskeletal rearrangements in the cell, however the functional role of plasma gelsolin is less well understood (Silacci et al., 2004). Osborn et al. (2007) suggested that a potential role is to act as a sequestering protein to buffer the effects of the bioactive mediators it binds including LPA. This is particularly interesting since levels of circulating gelsolin are depleted on inflammation or injury, such as lung injury (Lind et al., 1988; Christofidou-Solomidou et al., 2002), rheumatoid arthritis (Osborn et al., 2008), trauma (Dahl et al., 1999), liver failure, myocardial infarction, septic shock and myonecrosis (Suhler et al., 1997). This phenomenon may be of functional importance since administration of gelsolin to mice subjected to systemic sepsis improves survival significantly (Lee et al., 2007). Gelsolin is also thought to be necessary for the proper maintenance of vascular permeability (Becker et al., 2003). One study showed that plasma gelsolin can inhibit LPA-induced responses in platelets (Osborn et al., 2007), while another showed that LPA reduces the F-actin-severing ability of gelsolin (Meerschaert et al., 1998). The function of the interaction between LPA and gelsolin remains elusive, its contribution to the bioavailability of circulating LPA and the function of gelsolin are not well understood.

Gelsolin acting as an LPA-sequestering protein contrasts with the role of albumin acting as an LPA-presenting or carrier protein, though one study suggests that both serum albumin and gelsolin can enhance LPA-induced responses observed in a variety of assays on rat cardiac myocytes (Goetzl et al., 2000). How these LPA-binding proteins affect receptor-ligand interaction and activation is not yet known.

1.1.3. LPA metabolism

LPA is an important intermediate in the de novo synthesis of lipids and its many species are generated from a variety of precursors and involve a range of biosynthetic enzymes, though the relative contributions of the specific isoenzymes involved is not thoroughly understood. There are thought to be two main routes for LPA generation: it can be formed from lysophospholipids by a lysophospholipase D (lysoPLD; Figure 1.1B, Route 1) or, alternatively, phospholipids can be converted by a phospholipase D (PLD) to phosphatidic acid, which is then deacylated by a phospholipase A₁ or A₂ (PLA₁/₂) to form LPA (Figure 1.1B, Route 2). The first route is thought to be responsible for
extracellular LPA production, i.e. in serum and plasma, and the second for cellular LPA production, including that of platelets (Aoki et al., 2008). Notably, platelets are thought to account for a substantial proportion of LPA production (Aoki et al., 2002). Autotaxin has been identified as the lysoPLD present in human serum (Umezu-Goto et al., 2002; Tokumura et al., 2002) and is thought to be the key LPA-synthesising enzyme in the first pathway (Aoki et al., 2008). Two membrane-associated, phosphatidic acid-selective PLA₁ isoenzymes, mPA-PLA₁α and mPA-PLA₁β, have been identified as the key enzymes involved in the second pathway (Sonoda et al., 2002).

Autotaxin is a secreted enzyme (Pradere et al., 2007), while mPA-PLA₁αβ have been found to localise to detergent-resistant portions of the plasma membrane (Hiramatsu et al., 2003). Interestingly, mutations in the mPA-PLA₁α isoenzyme are linked to similar genetic hair-loss diseases (Kazantseva et al., 2006; Ali et al., 2007) to those linked with mutation of LPA₆ (Pasternack et al., 2008). These studies support the idea that mPA-PLA₁α is a physiologically relevant source of LPA in vivo, though autotaxin has been far more extensively studied. Autotaxin was first identified as a tumour cell motility factor (Stracke et al., 1992) and its effects were later attributed to its production of LPA when it was found to be the same lysoPLD present in serum (Umezu-Goto et al., 2002).

1.2. **G protein-coupled receptors**

1.2.1. **The G protein-coupled receptor (GPCR) superfamily**

LPA receptors belong to the G protein-coupled receptor (GPCR) superfamily. GPCRs are transmembrane receptors that couple extracellular ligand binding to intracellular effects via activation of G proteins, allowing the cell-cell communication that is necessary for the function of complex, multicellular organisms. They are the most abundant and diverse family of membrane receptors and are related by common structural characteristics; they consist of an extracellular N-terminus, seven transmembrane α-helices, linked by three intracellular and three extracellular loops, and an intracellular C-terminus (Figure 1.2A).

The GPCR superfamily was initially split into three sub-families (A, B and C) based on sequence homology and endogenous ligand specificity (Attwood and Findlay, 1994; Kolakowski, 1994). Family A is by far the largest and most varied. It includes
A, schematic illustrating the basic structural features common to the GPCR superfamily, including an extracellular N-terminal domain, seven transmembrane domains joined by intra- and extra-cellular loops and an intracellular C-terminal domain. B, schematic illustrating the ‘GTPase’ cycle which governs G protein activation. C, schematic illustrating the initial events of G protein activation by an agonist-bound GPCR.

Figure 1.2
rhodopsin and receptors for adrenaline and many neuropeptides, as well as lipid mediators, including the subject of this project, lysophosphatidic acid (Chun et al., 2010); family B comprises receptors for peptide hormones, including secretin, glucagon and calcitonin; and family C members are characterised by a very large extracellular ligand-binding domain and include the metabotropic glutamate and Ca$^{2+}$-sensing receptors (Pierce et al., 2002). The non-classical frizzled/taste2 and adhesion receptors are also now recognised as GPCR sub-families according to more recent phylogenetic analysis (Fredriksson et al., 2003).

The GPCR superfamily is one of the largest families of proteins in man, comprising over 800 members. Ligands for GPCRs are highly varied and include proteins, small molecules, lipids, peptides, nucleotides and even ions and photons (Fredriksson et al., 2003). Such variety allows these receptors to mediate responses to a wide array of environmental stimuli, allowing them to play a role in the regulation of a broad range of physiological processes. It has been estimated that over half of commercially available drugs target GPCRs (Flower, 1999) and they are the subject of many drug discovery programmes, making GPCRs of great clinical and therapeutic, as well as commercial, importance.

1.2.2. Guanine nucleotide-binding (G) proteins

GPCRs function at the interface of extracellular stimuli and intracellular responses and can be described as signal transducing receptors. When an extracellular ligand binds its cognate GPCR, the receptor undergoes conformational changes that result in the activation of intracellular effectors. The multiplicity of ligands for GPCRs somewhat contrasts with their relatively small repertoire of primary intracellular effectors; of these, guanine nucleotide-binding proteins (G proteins) were the first to be characterised.

G proteins are heterotrimeric proteins comprising $\alpha$, $\beta$ and $\gamma$ subunits. The $\beta$ and $\gamma$ subunits are closely associated through a tight, coiled-coil interaction and both $G\alpha$ and $G\beta\gamma$ moieties are linked to the membrane by post-translational modifications (Sondek et al., 1996). As with many guanine nucleotide-binding proteins, GTP binding and hydrolysis regulates the activity of the protein such that its activity can be switched 'on' and 'off' according to its GTP/GDP bound state (Figure 1.2B). In its activated state, $G\alpha$
is bound to GTP and dissociates from Gβγ; the GTP is then hydrolysed by the intrinsic GTPase activity of the Ga subunit to form the inactive, GDP-bound Ga which then re-associates with Gβγ, as shown in Figure 1.2C (Bourne et al., 1990).

The intrinsic GTPase activity of Ga can be facilitated by GTPase-activating proteins (GAPs), which enhance the often slow intrinsic catalytic activity of Ga. A large family of proteins possessing RGS (regulator of G protein signalling) domains have been characterised and are considered the major modifiers of Ga protein GTPase activity (Willars, 2006; Kach et al., 2012). Similarly, guanine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP and so enhance the activation of the G protein (Bourne et al., 1990). In this context, the ligand-bound GPCR acts as the GEF, promoting the formation of GTP-bound, activated G protein and dissociation of Ga-GTP from Gβγ. Both the Ga-GTP and Gβγ moieties have the potential to act as signalling proteins, affecting the activation state of downstream effectors.

There are multiple variants of each G protein subunit giving rise to the potential for hundreds of combinations. Over 20 variants of Ga are encoded by 16 genes, while there are 5 genes for Gβ-subunits and at least 12 for Gγ-subunits (Cabrera-Vera et al., 2003). This gives rise to hundreds of potential combinations of αβγ subunits. However, some subunit variants have a restricted tissue expression pattern, such that the possible number of combinations found physiologically is likely to be much lower (Milligan and Kostenis, 2006).

1.2.3. G protein-mediated signalling

Generally speaking, it is the Ga-subunit that dictates the coupling of the receptor to its downstream effectors. There are four families of Ga, which are grouped according to structural similarity: Gaα, Gaαo, Gaαq/11 and Gaα12/13 (Strathmann and Simon, 1991). Gaα was the first identified family and is characterised by its ability to stimulate the activation of the membrane-bound enzyme, adenylyl cyclase (AC), and therefore induce production of adenosine 3',5'-cyclic monophosphate (cAMP; Northup et al., 1980; Cerione et al., 1984). Gaαo was identified shortly afterwards and was found to inhibit AC and thus reduce [cAMP], (Bokoch et al., 1984). [cAMP], is also regulated by phosphodiesterases (PDEs), a family of enzymes that catalyse the breakdown of cAMP into 5'-AMP. Collectively, these mechanisms allow for fine control of [cAMP], and
thus activation of its downstream effectors. Of these, the cAMP-dependent protein kinase (PKA) is the best known. PKA is a tetrameric protein comprised of two regulatory and two catalytic subunits. In low [cAMP], conditions the regulatory subunits are bound to the catalytic subunits and sterically inhibit their activity. When [cAMP] increases, cAMP binds to the regulatory subunits, causing a conformational change and dissociation, thus liberating the catalytic subunits and increasing their activity. PKA activation can then go on to affect many and varied downstream targets, including elements of the cellular contractile apparatus and enzymes involved in glycogen breakdown. In this way, many GPCR ligands can activate production of a common second messenger, but give rise to differing effects in different cellular environments.

The Gαq/11 family can regulate the activity of another membrane-associated enzyme, phospholipase C (PLC). PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂), producing diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) which can both function as signalling molecules. While DAG stays within the plasma membrane, IP₃ is water-soluble and diffuses into the cytoplasm, allowing it to reach its effector, the IP₃ receptor (IP₃R) – a Ca²⁺ channel associated with the endoplasmic reticulum (ER) membrane. Similarly, the DAG released can also go on to effect activation of the Ca²⁺-dependent protein kinase (PKC). IP₃R activation by IP₃ binding causes release of Ca²⁺ from the intracellular stores of the ER, thus greatly increasing the [Ca²⁺]. Like cAMP, Ca²⁺ is an important second messenger that can go on to activate myriad downstream effectors, including proteins with a calmodulin domain. Where [cAMP], is regulated by synthesis and degradation, [Ca²⁺] is governed by tightly controlled storage and release (Berridge and Irvine, 1989; Lipp and Reither, 2011).

In 1991, a fourth family of Gα subunit, Gα₁₂/₁₃, was discovered by Strathmann and Simon. Both Gα₁₂ and Gα₁₃ proteins are ubiquitously expressed and share ~67 % amino acid sequence identity in mice (Strathmann and Simon, 1991). Many GPCRs couple to Gα₁₂/₁₃, including the receptors for LPA (Hains et al., 2006), however, all GPCRs thus far shown to couple Gα₁₂/₁₃, also couple to other heterotrimeric G proteins (Riobo and Manning, 2005; Siehler, 2008). Gα₁₂/₁₃ proteins can act via G proteins of the Ras superfamily, rather than by altering cellular concentrations of a small molecule mediator as for Gαᵣ, Gα₄₀ and Gα₉/₁₁. GTP-bound Gα₁₂/₁₃ stimulates the activity of a
RhoGEF thereby activating the small GTPase, RhoA. Gα12/13 has been shown to activate four RhoGEFs: p115-RhoGEF, PDZ-RhoGEF, LARG and Lbc-RhoGEF (Kozasa et al., 1998; Fukuhara et al., 1999; Fukuhara et al., 2000; Dutt et al., 2004). In its inactive state, GDP-bound RhoA is in the cytoplasm and its prenyl group, which allows membrane association, is masked by a guanine nucleotide dissociation inhibitor (GDI; Garcia-Mata et al., 2011). Following activation by Gα12/13, RhoGEF facilitates the exchange of GDP for GTP at RhoA. Dissociation of the GDI and translocation of RhoA to the plasma membrane is also required for RhoA activation. As well as being regulated by RhoGEFs and RhoGAPs, the activity of RhoA is regulated by GDIs and GDI dissociation factors. Therefore, RhoA activity can be finely controlled by a network of regulatory proteins. The best studied effectors of RhoA are the Rho-associated coiled-coil-containing protein kinases (ROCK; Siehler, 2009). Typical downstream effects of RhoA activation include cytoskeletal rearrangements, cell migration and formation of focal adhesions (Amano et al., 1996; Iwanicki et al., 2008).

Additionally, Gβγ subunits have been shown to mediate and regulate other downstream signalling events. The first example of signalling through Gβγ was in the regulation of Kir3 ion channels (Logothetis et al., 1987). Gβγ has since been shown to regulate the function of other ion channels including voltage-dependent Ca2+ channels and TRPM1 channels (Holz et al., 1986; Shen et al., 2012). Adenylyl cyclase isoenzymes are regulated by Gβγ, with some isoforms inhibited, and some stimulated by Gβγ interactions (Tang and Gilman, 1991). Gβγ can bind to pleckstrin homology (PH) domains on some PLC isoenzymes to regulate their activity (Wang et al., 2000). Some groups have suggested that different pairings of Gβ and Gγ differentially regulate PLC (Wing et al., 2001). Gβγ has also been shown to regulate the function of phosphoinositide 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (Kurosu et al., 1997; Koch et al., 1994).

1.2.4. G protein-independent signalling

It is now recognised that in addition to signalling via G proteins by these classical pathways, GPCRs interact with and affect the function of other proteins. In return, many proteins affect the function of GPCRs and the concept of a “signalosome” has developed. Some such interactions can give rise to their own signalling pathways, independent of G protein activation.
The best characterised of these effectors are the arrestins. Of this small family of four proteins, arrestin-1 and arrestin-4 are exclusively expressed in photoreceptor cells (Craft et al., 1994), while arrestin-2 (β-arrestin1) and arrestin-3 (β-arrestin2) have near ubiquitous expression (Sterne-Marr et al., 1993). The non-visual arrestins play a pivotal role in the desensitisation, internalisation and recycling of GPCRs throughout the body (Pierce and Lefkowitz, 2001). For a GPCR to be desensitised it is firstly phosphorylated at the C-terminus and/or intracellular loops by a G protein-coupled receptor kinase (Benovic et al., 1989). Other kinases, such as PKA and PKC, can also phosphorylate GPCRs, which can contribute to desensitisation (Lefkowitz et al., 1990). Arrestin is then recruited to the phosphorylated GPCR, thereby sterically hindering G protein interaction and causing receptor desensitisation (Krupnick et al., 1997). Arrestin then mediates receptor internalisation via endocytosis of clathrin-coated vesicles. From these endocytic vesicles, the receptor can be recycled back to the plasma membrane or targeted for degradation. Many factors, including the extent of receptor phosphorylation and strength of GPCR-arrestin interaction, determine the pathway taken (Luttrell and Lefkowitz, 2002; Tobin et al., 2008).

In more recent years, it has become clear that this is merely one facet of β-arrestin function. β-arrestins can act as scaffolds for many downstream signalling proteins in response to GPCR activation and therefore act as signal transduction components in their own right. The best studied example of this is activation of the extracellular signal-regulated kinase (ERK) by the recruitment of c-Src and β-arrestin to ligand-bound β2-adrenoceptors (Luttrell et al., 1999). Further study, including application of a proteomics approach, has shown that β-arrestins interact with a wide variety of proteins and it is becoming clear that β-arrestins can orchestrate a diverse range of effects downstream of GPCR activation (Xiao et al., 2007). It has been demonstrated that β-arrestin-signalling, independent of classical G protein-signalling, mediates in vivo bone formation showing that such pathways are of physiological significance (Gesty-Palmer et al., 2009).

Other proteins have also been shown to be involved in GPCR regulation. Receptor activity-modifying proteins (RAMPs) are type 1 transmembrane proteins that interact with certain family B GPCRs. RAMPs have been shown to regulate receptor trafficking, signalling and, remarkably, receptor pharmacology (Sexton et al., 2006).
Another group of GPCR-interacting proteins are characterised by possession of a PDZ domain. There are estimated to be 200-300 PDZ domain-containing proteins; they are cytoplasmic proteins that can contain several PDZ domains and many contain other canonical protein interaction domains (Lee and Zheng, 2010). PDZ proteins act as scaffolding molecules and varied functions of GPCR-PDZ protein interactions have been demonstrated. For example, expression of a PDZ protein can switch type 1 parathyroid hormone receptor (PTH1R) signalling from the adenylyl cyclase pathway to the PLC pathway (Mahon et al., 2002), while the PDZ protein, NHERF1, mediates β2-adrenoceptor regulation of the sodium-proton exchanger, NHE3 (Hall et al., 1998a; Hall et al., 1998b). Roles in receptor trafficking have also been shown; PDZ proteins have been shown to promote recycling of receptors (Gage et al., 2005), or retention of receptors at the cell surface (Katsushima et al., 2013).

As is becoming evident, activation of a G protein by a GPCR is merely the first layer of the effects of receptor activation. Each of these pathways leads to the generation of second messengers, which in turn have their own effectors and a complex network of ‘crosstalk’ can exist between these pathways (Pierce et al., 2002; Rang et al., 2003). GPCRs do not function in isolation, but instead interact with many signalling and scaffolding proteins. Increasingly, spatial aspects of signalling events are being considered, in addition to kinetic parameters and measures of the magnitude of response.

1.2.5. Tools for studying GPCR-G protein-dependent signalling

Despite intense research and interest in GPCR-G protein transduction, remarkably few robust pharmacological tools exist for their study. The initial identification of Ga\textsubscript{i0} and Ga\textsubscript{s} was enabled by their susceptibility to the exotoxins of Bordetella pertussis and Vibrio cholerae, pertussis toxin (PTx) and cholera toxin (CTx), respectively (Milligan and Kostenis, 2006). Both toxins function by transferring an ADP-ribosyl group onto a specific amino acid residue in their target Gα subunit. In the case of PTx, ADP-ribosylation of Ga\textsubscript{i0} inhibits G protein activation by GPCRs (Gill and Meren, 1978) and thus curtails its ability to inhibit adenylyl cyclase activity. On the other hand, CTx ADP-ribosylates Ga\textsubscript{s}, which inhibits the intrinsic GTPase activity of Ga\textsubscript{s} such that it remains in an active, GTP-bound state (Cassel and Pfeuffer, 1978). These basic tools have greatly facilitated advances in our understanding of GPCR signalling.
Unfortunately, such practicable tools are not available for the other Gα subtypes. Instead, expression of inhibiting mini-genes and complex immuno-precipitation methods can be used to attribute a function to a particular GPCR-G protein coupling (Gilchrist et al., 2001; Mistry et al., 2011). More commonly, downstream signalling events are used to imply G protein-coupling.

For Gα_{q/11}-coupled GPCRs, Ca^{2+} mobilisation can be readily visualised using ratiometric Ca^{2+}-sensitive dyes, such as FURA-2-AM and FLUO-4-AM, in concert with epi-fluorescence microscopy (Paredes et al., 2008). Additionally, PLC involvement can be demonstrated using [^3H]inositol labelling of cells and subsequent anion-exchange chromatography to recover the inositol phosphate fraction (Skippen et al., 2013). While Gα_{q/11}-specific antagonists have been developed (Takasaki et al., 2004), these are not well characterised and are not commercially available.

For Gα_{i/o} and Gα_{s}, changes in [cAMP], can be measured by a number of assays, including a[^3H]adenine prelabelling assay (Shimizu et al., 1969) and a cAMP/[^3H]cAMP binding-protein saturation method (Brown et al., 1971). For high-throughput applications, chemiluminescence proximity assays have become popular. This is a variation of a cAMP/labelled cAMP binding-protein saturation method wherein biotinylated cAMP, streptavidin-coated donor beads and cAMP antibody-coated acceptor beads are used for detection (Golla and Seethala, 2002). Other cAMP assays based on FRET, enzyme complementation or electro-chemiluminescence have also been developed (Hill et al., 2010).

Assessment of Gα_{12/13} involvement presents different difficulties as the pathway involves activation of a protein, rather than alteration of the concentration of a mediator in the cell. Initial assays relied on measurement of[^35S]GTPγS binding alongside immunoprecipitation of either RhoA or Gα_{12/13} (Barr et al., 1997). An alternative method exploits the specificity of the RhoA-binding domain of Rhotekin, a downstream effector of RhoA. This allows specific detection of GTP-bound RhoA and prevents GTP-hydrolysis in the sample (Ren and Schwartz, 2000). A recombinant, GST-tagged version of the RhoA-binding domain is used to immunoprecipitate GTP-bound RhoA using glutathione-coated beads. More recent attempts to measure RhoA activation have used biosensors and FRET to detect active RhoA (Yoshizaki et al., 2003; Pertz et al.,
and eGFP-tagged RhoGEFs to visualise translocation to the plasma membrane (Meyer et al., 2008).

1.3. **LPA receptors**

Lysophosphatidic acid is a major serum phospholipid and bioactive lipid mediator which exerts a broad range of effects through its family of cognate GPCRs. LPA receptors have been shown to couple to multiple G proteins, including members of the Gαi/o, Gαq/11, Gα12/13 and Gαs families (Contos et al., 2000; Takuwa et al., 2002; Noguchi et al., 2003; Lee et al., 2007), and can effect a variety of cellular functions in a range of tissues and cell-types. LPA receptor-mediated effects include stimulation of cell migration (Mukai et al., 2000; Kim et al., 2008), cell proliferation (van Corven et al., 1989; van Leeuwen et al., 2003), cell survival (Song et al., 2005; Chen et al., 2008) and morphological changes, including neurite retraction and elongation (Jalink et al., 1993; Ishii et al., 2000). LPA-mediated signalling may contribute to physiological processes including wound healing (Watterson et al., 2007; Prestwich et al., 2008), neurogenesis (Fukushima et al., 2007; Matas-Rico et al., 2008), angiogenesis (Rivera-Lopez et al., 2008), reproduction (Hama et al., 2007; Ye, 2008) and cancer cell proliferation and metastasis (Mills and Moolenaar, 2003; Yu et al., 2008). Pathways implicated in LPA receptor-mediated cell signalling are varied, as would be predicted from the diversity of G protein-coupling partners, and include Gαq/11-mediated activation of phospholipase C and Ca2+ mobilisation (Ohata et al., 1997; Zhou et al., 1999), Gαi/o-mediated inhibition of adenylyl cyclase and reduced cAMP levels (Fukushima et al., 1998), activation of the Ras/mitogen-activated protein kinase (MAPK) cascade (van Corven et al., 1993), Gα12/13-mediated Rho activation and cytoskeletal rearrangements (Bian et al., 2006; Lee et al., 2006), phosphoinositide-3-kinase (PI3K) mediated signalling (Yart et al., 2002), and Gαs-mediated activation of adenylyl cyclase and consequent increase in cAMP levels (Noguchi et al., 2003; Lee et al., 2006). Clearly, LPA signalling is pleiotropic, having the potential to exert actions via many mechanisms to produce its diverse effects.

1.3.1. **The lysophosphatidic acid receptor family**

Evidence for LPA being biologically active started to emerge in the late 1970s, when it was found to affect blood pressure (Tokumura et al., 1978) and induce platelet
**Figure 1.3**

A schematic showing LPA receptor G protein coupling. Dashed lines indicate where evidence is contentious or preliminary.
aggregation (Gerrard et al., 1979). Further study revealed that LPA was able to induce a variety of other cellular responses, though it wasn’t until the discovery that LPA was the natural ligand for a family of GPCRs some 20 years later that the mechanism underlying LPA’s biological effects could begin to be unravelled (Hecht et al., 1996; Erickson et al., 1998; Fukushima et al., 1998). These receptors were formerly known as the endothelial differentiation gene (Edg) receptors and belong to the same family as several GPCRs for another lipid mediator, sphingosine-1-phosphate (Chun et al., 2002). Since their discovery, roles for the LPA receptors have been demonstrated in many biological functions and are being actively researched by groups from a range of fields and backgrounds. The LPA receptor family has been steadily growing since the identification of what is now referred to as LPA1 in 1996, with new members suggested as recently as 2008 (Hecht et al., 1996; Pasternack et al., 2008; Alexander et al., 2011). Most studies to date have focussed on LPA1-3 and relatively little has been published on the newer family members, though it should be noted that many functions of LPA have not yet been attributed to any particular receptor subtype(s), owing to the paucity of pharmacological tools available for the study of LPA receptor subtypes.

It is interesting to note that the newer LPA receptor subtypes, LPA4, LPA5 and LPA6, are structurally more closely related to P2Y purinergic receptors than to the so-called Edg family LPA receptors (LPA1, LPA2 and LPA3) and S1P receptors. LPA4, LPA5 and LPA6 are thought to have evolved from a distinct lineage to the Edg family LPA receptors (Yanagida and Ishii, 2011).

1.3.1.1. LPA1

LPA1 was initially identified because its gene, formerly known as ventricular zone gene 1, is highly expressed in neurogenic regions of the developing brain. Study of the gene product showed that it is a GPCR for LPA (Hecht et al., 1996). LPA1 has been shown to couple to multiple G proteins: Ga10, Ga11 and Ga12/13 (Fukushima et al., 1998; Ishii et al., 2000). Its expression is widespread, but is enriched in the brain during development and LPA1-null mice show many signs of altered cortical development (An et al., 1998; Estivill-Torrus et al., 2008; Ohuchi et al., 2008). At a cellular level, LPA1 activation typically leads to cell proliferation, migration, survival and cytoskeletal rearrangements (Fukushima et al., 1998; Weiner and Chun, 1999; Deng et al., 2002; Shano et al., 2008; Komachi et al., 2009).
LPA₁ has been implicated in a broad range of physiological and pathophysiological processes. Roles for LPA₁ have been demonstrated for several processes in neurological development (Pilpel and Segal, 2006; Fukushima et al., 2007; Estivill-Torrus et al., 2008; Matas-Rico et al., 2008) and it has been suggested that LPA₁ deletion may serve as a mouse model for schizophrenia, or other psychiatric disease (Harrison et al., 2003; Roberts et al., 2005). LPA₁ has been implicated in the myelination process in both the peripheral and central nervous system and there is thought to be LPA₁ dysfunction in demyelination, which can cause neuropathic pain (Weiner et al., 1998; Contos et al., 2000; Xie et al., 2008; Halder et al., 2013).

The pro-proliferative and migratory effects of LPA are thought to be dysregulated in a number of conditions and LPA₁ has been implicated in cancer, renal and pulmonary fibrosis, cardiac hypertrophy, intimal hyperplasia in atherosclerosis and mesenchymal stem-cell recruitment to joints in rheumatoid arthritis (Hama et al., 2004; Chen et al., 2008; Panchatcharam et al., 2008; Pradere et al., 2008; Tager et al., 2008; Song et al., 2010). Understandably, LPA₁ is being pursued as a therapeutic target by several fields, though it should be noted that effects of LPA₁ activation are not consistent across all cell types. For example, there are some reports of LPA₁ mediating protection from tumourigenesis (Kato et al., 2012).

1.3.1.2. LPA₂

LPA₂ was identified by its sequence similarity to LPA₁ (Contos and Chun, 2000) and has been shown to couple to Ga₁₂₀, Ga₄₁₁ and Ga₁₂/₁₃ proteins (Ishii et al., 2000). The expression pattern of LPA₂ is somewhat more restricted than LPA₁, being particularly high in leukocytes and testis (An et al., 1998). Lpar2 knockout mice are grossly normal and many of the physiological functions of this receptor subtype remain to be elucidated, though experiments on cells from Lpar1/Lpar2 double knockout mice suggest that LPA₂ might have a role in supporting normal LPA₁ function (Contos et al., 2002). Demonstrated functions of LPA₂ activation include cell migration and survival, and LPA₂ has been most intensively studied in cancer, particularly gynaecological cancers (Zheng et al., 2001; Deng et al., 2002). LPA₂ is up-regulated in ovarian cancer cells and LPA₂ expression has been linked to invasiveness of ovarian cancer cell tumours in a xenograft mouse model (Yu et al., 2008; Goetzl et al., 1999). LPA₂ siRNA knockdown reversed the pro-migratory effects of LPA stimulation in an endometrial
cancer cell line and demonstrated an LPA_2-dependent secretion of matrix metalloproteinase 7 (Hope et al., 2009).

Investigations into LPA_2 signalling indicate that it interacts with several other signalling components. LPA_2 has been shown to interact with several PDZ domain-containing proteins, including NHERF2, MAGI-3, LARG and PDZ-RhoGEF (Oh et al., 2004; Yamada et al., 2005; Zhang et al., 2007; Lin and Lai, 2008). As an example, LPA_2 interacts with thyroid receptor-interacting protein 6 (TRIP6), a PDZ domain-containing member of the zyxin family of proteins, which are implicated in formation of focal adhesions. This interaction is thought to regulate LPA-induced cell migration and changes to cell morphology in a process regulated by phosphorylation and dephosphorylation of TRIP6 (Lai et al., 2005; Lai et al., 2007). Additionally, LPA_2 has been shown to regulate EGF receptor activity via a Gα_{12/13}/Rho-dependent mechanism in pancreatic cancer cells (Komachi et al., 2009).

### 1.3.1.3. LPA_3

LPA_3 couples to Gα_q/11 and Gα_i/o, but coupling to Gα_{12/13} has not been demonstrated as for some other LPA receptors (Anliker and Chun, 2004). LPA_3 is potently activated by less physiologically common LPA species with unsaturated 2-acyl-linked fatty acid groups (Bandoh et al., 2000). Such LPA species would be generated by a type-1 phospholipase A (PLA_1), though a clear functional link has not yet been established between this enzyme family and the LPA_3 receptor, as has been found for LPA_6 and mPA-PLA_1α/β (see below).

A key area of focus for LPA_3 research is in female reproduction and embryo implantation. LPA_3 deficient knockout mice appear broadly normal, but the females have reproductive abnormalities, including delayed and irregular implantation, prolonged gestation and reduced litter size (Ye et al., 2005; Hama et al., 2007). Though its expression pattern is relatively restricted, LPA_3 is expressed, not only in gynaecological tissues, but also in heart, lung, kidney, pancreas and testis and it is likely that more physiological functions of this receptor-subtype will emerge (Bandoh et al., 1999; Chen et al., 2008).
**LPA_4**

LPA_4_ was initially identified when it was found to respond specifically to LPA in a project designed to discover the function of orphan GPCRs using radioligand binding (Noguchi _et al._, 2003). Two studies published at the same time using the LPA receptor-null B103 cell-line as a model background for pharmacological investigation of exogenously expressed LPA_4_ provided conflicting data regarding the G protein coupling of this receptor (Lee _et al._, 2007; Yanagida _et al._, 2007). Both papers reported Gα_12/13_-mediated neurite retraction and cell rounding in LPA_4_-expressing B103 cells, using either Gα_12_ and Gα_13_ mini-genes to disrupt coupling (Lee _et al._, 2007), or the ROCK inhibitor Y-27632 (Yanagida _et al._, 2007). However, despite both groups using the same cell background in which to express LPA_4_, Lee _et al._ (2007) reported a four-fold increase in cellular cAMP levels in response to 1 µM LPA, which was completely blocked by expression of a Gα_s mini-gene, whereas Yanagida _et al._ (2007) observed no increase in cAMP levels above basal, even at 10 µM LPA. Further to this, Lee _et al._ (2007) found that the Ca^{2+} response observed in LPA_4_-expressing B103 cells in response to 1 µM LPA had a Gα_q/11_- and Gα_i/o_-mediated component, whereas Yanagida _et al._ (2007) reported that this response could be completely blocked by the Gα_q/11_ protein-specific inhibitor YM-254890. Clearly, further work is needed to fully elucidate the downstream signalling responses elicited by this receptor. Studies of _Lpar4_ knockout mice and zebrafish treated with morpholino-antisense oligonucleotides against _Lpar4_, suggest a critical role for LPA_4_ in vascular development, which may involve cooperation with LPA_1_ (Sumida _et al._, 2010; Yukiura _et al._, 2011).

**1.3.1.4. LPA_5**

After the identification of LPA_4_, LPA was found to be an activating ligand of the closely related orphan receptor GPR92, which was proposed to be another LPA receptor (Kotarsky _et al._, 2006). Follow-up investigations into this orphan receptor revealed that LPA induced a range of responses, including neurite retraction, stress fibre formation, cAMP accumulation and Ca^{2+} responses in cells exogenously expressing GPR92. Additionally, transfection with GPR92 increased specific [³H]LPA binding approximately 3.5-fold over wild-type levels in B103 cells (Lee _et al._, 2006). There has been some debate regarding the most potent endogenous lipid agonist for this receptor, with another group reporting that N-arachidonylglycine (NAG) displays similar, and
farnesyl pyrophosphate greater, potency at this receptor (Oh et al., 2008). A claim refuted by a further investigation that confirmed LPA as the dominant endogenous ligand (Williams et al., 2009). Farnesyl pyrophosphate has also been identified as a ligand for LPA3 and can inhibit LPA-induced Ca\textsuperscript{2+} mobilisation in RH7777 cells over-expressing LPA3 (Liliom et al., 2006). While LPA might be the dominant ligand for GPR92/LPA\textsubscript{5}, the physiological role of LPA receptor activation by endogenous farnesyl pyrophosphate and other lipids remains largely unexplored. Liliom et al. (2006) have suggested that they might represent endogenous modulators for LPA receptors. GPR92 has now formally been designated LPA\textsubscript{5} by the International Union of Basic and Clinical Pharmacology (Davenport et al., 2013). LPA\textsubscript{5} couples to Go\textsubscript{q/11} and Ga\textsubscript{12/13} protein families (Lee et al., 2006).

LPA\textsubscript{5} shows preference for alkyl-linked LPA species (Williams et al., 2009), while LPA\textsubscript{1}, LPA\textsubscript{2} and LPA\textsubscript{3} prefer acyl-linked species (Bandoh et al., 2000; Tokumura et al., 2002). Early studies showed that LPA-induced responses in platelets are more potent for alkyl-linked LPA species and this prompted the hypothesis that there is a platelet-type LPA receptor (Simon et al., 1982; Tokumura, 1995). It now seems likely that LPA\textsubscript{5} is the platelet-type LPA receptor since LPAR5 mRNA is highly expressed in platelets, and RNAi knockdown of LPA\textsubscript{5} in megakaryocytic cell lines attenuates LPA responses (Amisten et al., 2008; Khandoga et al., 2011). This is also backed up by pharmacological studies (Khandoga et al., 2008; Williams et al., 2009).

While LPA\textsubscript{1} and LPA\textsubscript{2} have been shown to mediate the chemoattractant properties of LPA and contribute to invasion and metastasis in cancer models (Zheng et al., 2001; Komachi et al., 2009), LPA\textsubscript{5} may mediate the chemorepellent roles of LPA. LPA\textsubscript{5} was shown to mediate LPA- and autotaxin-induced chemorepulsion in certain cancer cell-lines using siRNA receptor knockdown. As observed for LPA\textsubscript{5}-mediated responses in platelets, such responses were more potently activated by alkyl-linked LPA species (Jongsma et al., 2011). This could have consequences for research targeting LPA signalling for cancer therapy.

1.3.1.5. LPA\textsubscript{6}

The orphan receptor 6H1 was initially identified as a purinoceptor and termed P2Y\textsubscript{5} (Webb et al., 1996), though it was later reported that nucleotides did not induce any
second messenger responses that are typical of this family and the receptor was re-orphaned (Li et al., 1997). When a nonsense mutation of P2RY5 was found to be responsible for the hair loss disease, hypotrichosis simplex, in a consanguineous family, LPA was identified as an agonist using CRE-luciferase reporter and radioligand binding assays (Pasternack et al., 2008). After some initial debate (Lee et al., 2009; Yanagida et al., 2009), this receptor has now been accepted as a sixth LPA receptor subtype (Davenport et al., 2013). LPA₆ is thought to couple to the Gα₁₂/₁₃ protein family (Yanagida et al., 2009).

Its role in hair development is the best characterised function of LPA₆ and has provided some interesting insights into the activation of this LPA receptor family member. Shortly before identification of LPAR6 as a causative gene for hypotrichosis simplex, mutations in the gene encoding a membrane-associated enzyme that produces 2-acyl LPA species, mPA-PLA₁, were found to cause the same autosomal-recessive hair growth disorder (Kazantseva et al., 2006). Both genes are expressed in the hair root sheath and experiments using knockout mice evidence a role for both genes in normal hair growth (Inoue et al., 2011; Shimomura, 2012). As for LPA₃, 2-acyl LPA species, like those produced by mPA-PLA₁, are more potent at LPA₆ than the more commonly occurring 1-acyl linked species (Yanagida et al., 2009; Inoue et al., 2011).

1.3.1.6. Other proposed LPA receptors

In addition to the more recently proposed LPA₄-₆ receptors, it has also been suggested that GPR87, GPR35 and P2Y₁₀ might represent new lysophospholipid receptors (Tabata et al., 2007; Murakami et al., 2008; Davenport et al., 2013; Zhao and Abood, 2013). Further confirmatory data are required to establish these receptors as bona fide LPA receptors. For example, LPA and kynurinic acid have both been proposed to be endogenous ligands for GPR35 (Barth et al., 2009; Yang et al., 2010). As for LPA₃ and LPA₆, 2-acyl LPA species have been found to be much more potent agonists at GPR35 than the more physiologically common 1-acyl LPA species (Oka et al., 2010). Clearly, this new area of LPA signalling is not well understood or characterised and it seems likely that new candidate receptors will continue to emerge. This presents some difficulties since the effects of existing LPA receptor ligands on these receptors and what contribution they make to the LPA response of a particular cell-type or tissue is largely unknown.
1.3.2. **The LPA receptor-autotaxin axis**

Though there are several enzymes that are able to generate LPA, autotaxin (ATX) is accepted as the major source of LPA production, since depletion of ATX in serum ablates LPA production (Tanaka *et al.*, 2006; Tsuda *et al.*, 2006). ATX knockout animals have a markedly more severe phenotype than any of the LPA receptor knockouts; embryos die at E9.5-E10.5 due to vascular defects, while heterozygous ATX knockouts have approximately half normal survival rates and plasma LPA levels (van Meeteren *et al.*, 2006; Tanaka *et al.*, 2006). LPA1 knockout mice have partial lethality, due to poor suckling in the pups, caused by defects in olfaction and some craniofacial dysmorphism (Contos *et al.*, 2000); LPA2 knockouts have no obvious phenotypic changes (Contos *et al.*, 2002) and female LPA3 knockouts have altered embryo spacing and delayed implantation (Hama *et al.*, 2007). LPA1/2/3 triple knockout mice have few additional phenotypic abnormalities than the sum of the single receptor knockouts (Ye *et al.*, 2008). The corollary of these data is that ATX contributes substantially to LPA production. Since the phenotype of ATX knockout is so much more severe than the LPA1/2/3 triple knockout, a substantial proportion of *in vivo* LPA function must be attributable to other entities such as LPA4/5/6, potentially as yet undiscovered LPA receptors, and/or non-receptor-mediated LPA or ATX effects. Some groups are now pursuing ATX as a therapeutic target, as altering its activity has the potential to modify signalling via all LPA receptor subtypes.

Some of the most prominent advances in LPA research during the course of my studies have concerned ATX structure-function and the crystal structure of ATX has interesting implications for LPA receptor function (Hausmann *et al.*, 2011; Nishimasu *et al.*, 2011). ATX is structurally and genetically identified as a member of the ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) family. Like the most closely related ENPPs, ENPP1 and ENPP3, ATX comprises a catalytically inactive C-terminal nuclease-like domain, a central phosphodiesterase domain and two N-terminal somatomedin B (SMB)-like domains. ATX is unique among ENPPs in that it is able to act as a lysophospholipase D and is secreted, while other ENPPs are single transmembrane domain-containing ecto-enzymes (Stefan *et al.*, 2005). ENPP1 and ENPP3, both act to convert ATP to pyrophosphate and are thought to have roles in tissue calcification and bone mineralisation (Stefan *et al.*, 2005).
18 amino acids, found near the catalytic site in other ENPPs, allows ATX to form a deep hydrophobic binding pocket of optimal dimensions to accommodate a 14:0 acyl group, thus permitting its divergent substrate specificity (Hausmann *et al.*, 2011; Nishimasu *et al.*, 2011). Mutagenesis studies have shown that absence of the 18 amino acids is necessary for lysophosphatidylcholine binding (Nishimasu *et al.*, 2011). Crystal structures of mouse ATX complexed with various LPAs show that the hydrophobic pocket is able to accommodate acyl chains of varying length in different conformations, for example, by allowing saturated chains to adopt a straight conformation and unsaturated chains to bend at their double bonds (Nishimasu *et al.*, 2011). In addition to this hydrophobic pocket, the crystal structure unexpectedly revealed a hydrophobic channel between the SMB1 and catalytic domains, which is linked to the active site and hydrophobic pocket (Hausmann *et al.*, 2011; Nishimasu *et al.*, 2011). It has been postulated that this channel is used for product delivery to LPA receptors (Nishimasu *et al.*, 2012).

The resolution of the ATX crystal structure has also provided insight into its potential interacting partners. The SMB domain of vitronectin has been shown to mediate vitronectin binding to plasminogen activator inhibitor-1 (PAI-1) to alter its function (Zhou, 2007). While the residues that mediate interaction of vitronectin's SMB domain with PAI-1 are not conserved in ATX (Hausmann *et al.*, 2011), this has led to speculation regarding the potential for ATX’s two SMB domains to mediate binding of ATX to other proteins (Tabchy *et al.*, 2011). Since the SMB domains interact extensively with the catalytic domain, this could enable such interacting partners to directly affect ATX substrate binding, catalytic function and/or localisation (Hausmann *et al.*, 2011). ATX has been shown to bind to the surface of activated platelets, lymphocytes and oligodendrocytes (Fox *et al.*, 2003; Kanda *et al.*, 2008; Pamuklar *et al.*, 2009). In the case of ATX-platelet binding, this has been shown to be β3-integrin-dependent (Pamuklar *et al.*, 2009) and is likely mediated by the SMB2 domain of autotaxin in an RGD motif-independent fashion (Hausmann *et al.*, 2011). Such findings have prompted several groups to suggest that the SMB2 domain of autotaxin interacts with integrins to localise LPA production (Moolenaar and Perrakis, 2011; Tabchy *et al.*, 2011; Nishimasu *et al.*, 2012; Hausmann *et al.*, 2013). One group has gone further to suggest that integrin binding by SMB2 could regulate conformational changes of the SMB1 domain, which is thought to be relatively flexible, and that such conformational
changes could cause opening of the hydrophobic channel (Hausmann et al., 2011). Such a mechanism could allow integrin binding to regulate both cell-surface localisation of autotaxin and product release, allowing highly localised production and delivery of LPA to its target receptors in a manner akin to substrate channelling in enzyme systems (Miles et al., 1999), although further work is required to validate this theory. This has interesting implications for LPA receptor pharmacology and it looks likely that advances in our understanding of autotaxin and its structural relationship with LPA receptors will be necessary for complete understanding of LPA receptor function.

1.3.3. **Physiological and pathophysiological roles for LPA and its receptors**

Since several LPA receptors are expressed almost ubiquitously (An et al., 1998) and each is able to activate a number of signalling pathways via interaction with a range of Gα protein subunits, it is predictable that LPA and its signalling have been implicated in a wide range of physiological and pathophysiological processes. LPA-induced effects have been detected and investigated in a wide variety of cell-types and well established responses include cell proliferation (Kim et al., 2006), differentiation (Fukushima et al., 2007), morphological changes, including neurite retraction (Tigyi et al., 1996a; Tigyi et al., 1996b), cell migration (Kim et al., 2008), increased cell survival (Ye et al., 2002) and chemokine generation (Lin and Boyce, 2005). Clearly many of these responses are mitogenic and roles for LPA in wound healing (Lee et al., 2000) and development (Choi et al., 2008) are being actively investigated. Several groups focus on the inappropriate or excessive stimulation of such responses, which can contribute to fibrosis (Pradere et al., 2008) or transformation and cancer (Mills and Moolenaar, 2003); LPA has been particularly implicated in the aetiology of ovarian cancer (Fang et al., 2002). Some of the LPA-induced responses relate more to a role in inflammation and/or immune responses (Graler and Goetzl, 2002), and LPA is also implicated in rheumatoid arthritis (Song et al., 2010) and airways inflammation (Zhao and Natarajan, 2009). The expression, regulation and signalling events mediated by LPA receptors in cells of the respiratory system will be the focus of my project, so the role of LPA in this system will be further discussed here.
1.4. **LPA and its receptors in respiratory physiology and disease**

Since LPA is implicated in signalling associated with inflammatory, proliferative and migratory responses, it is perhaps unsurprising that a growing body of evidence suggests a role for LPA in the molecular aetiology of respiratory diseases including asthma. Here, I will briefly introduce asthma and then go on to discuss how LPA has been implicated in its major components - airway inflammation, airway remodelling and hypercontractility. Though this project focuses on asthma, it should be noted that there is substantial overlap in the molecular and cellular "symptoms" of several respiratory conditions, including COPD and pulmonary fibrosis. Inflammation and remodelling are particularly common themes.

1.4.1. **Asthma**

The Global Initiative for Asthma (GINA) report provides the following description for asthma,

*Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airways hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment* (GINA report, 2012).

This offers a description of symptoms and features, rather than a definition of pathogenesis, drawing attention to the complexity of disease and the gaps in current understanding. This description highlights three key features of asthma; airway inflammation, airway hyperresponsiveness (AHR) and airflow obstruction.

The airway inflammation observed in asthma is chronic and present even in mild disease and in the absence of symptoms/exacerbations (Busse and Lemanske, 2001). There is infiltration of mast-cells, lymphocytes and eosinophils to the airways (Kraft *et al.*, 1996; Haley *et al.*, 1998; Vignola *et al.*, 1998). Asthmatic inflammation tends
towards an IgE/T_{H2} phenotype, such as would normally be induced against parasites (Burrows et al., 1989; Wills-Karp et al., 1998). Expression of T_{H2} inflammatory mediators, including IL-4, IL-13, IL-5 and GM-CSF, are substantially increased in the airway, but the T_{H1} mediator, IFN\(\gamma\), can also be elevated so there is contribution from both T_{H1} and T_{H2} inflammation (Chung and Barnes, 1999). It has been suggested that T_{H1}/T_{H2} imbalance early in life can contribute to asthma pathogenesis (Prescott et al., 1998).

In addition to inflammation, the asthmatic airways also undergo extensive remodelling. This is the term generally applied to the many structural changes observed that include an increase in smooth muscle mass, goblet cell hyperplasia, mucus gland hypertrophy, damage to the epithelium, sub-epithelial fibrosis with activation of myo-fibroblasts and increased deposition of extracellular matrix proteins (Benayoun et al., 2003; Bai and Knight, 2005; James et al., 2012). The overall effect of these features is airway narrowing/obstruction; increases in ASM mass and fibrosis are considered particularly important in this respect (Berair et al., 2013).

In the UK, 5.4 million people receive treatment for asthma and the NHS spends approximately £1 billion each year providing asthma care (AsthmaUK, 2010). However, disease presentation is highly variable and while severe asthmatics account for only 5-10\% of the asthmatic population, they account for approximately 50\% of hospital admissions (Bateman et al., 2008; Bousquet et al., 2010). A recent focus of asthma research has been in trying to better define asthma sub-groups and there are hopes that this will allow personalised therapy that will lead to better control of disease. Notably, degree of inflammation does not necessarily correlate with asthma symptoms (Haldar et al., 2008; Ingram and Kraft, 2012).

1.4.2. Production of LPA and expression of LPA receptors in the airways

Numerous studies have shown that a variety of LPA receptors are expressed in lung cells and tissues. Primary cultured human bronchial epithelial cells and cultured human airway smooth muscle cells express LPA_1, LPA_2 and LPA_3 mRNA (Ammit et al., 2001; Saatian et al., 2006). In human lung fibroblasts, mRNA for LPA_1, LPA_2, LPA_3, LPA_4 and LPA_5 was detected, with LPA_1 most highly expressed (Tager et al., 2008). Expression of the more newly identified receptors, LPA_4 LPA_5 and LPA_6, has not yet
been fully assessed. Additionally, LPA has been shown to elicit cellular responses in eosinophils, monocytes, mast cells, neutrophils and lymphocytes (Bagga et al., 2004; Idzko et al., 2004; Itagaki et al., 2005; Gustin et al., 2008; Zhang et al., 2012).

The LPA receptors expressed in the airway are likely to be active, since broncho-alveolar lavage (BAL) fluid contains a range of LPA species (Georas et al., 2007). Notably, LPA levels in BAL fluid are increased in some airway conditions, including following allergen challenge in asthmatics and a murine model of asthma, pulmonary fibrosis and exposure to lipopolysaccharide (Georas et al., 2007; Tager et al., 2008; Zhao et al., 2009; Zhao et al., 2011a; Zhao et al., 2011b). Primary cultured human bronchial epithelial cells express autotaxin (ATX) and the lipid phosphate phosphatase, LPP1, so lung epithelia is able to regulate lung LPA levels (Zhao et al., 2005; Zhao et al., 2011a). It is likely that ATX is the main source of LPA at sites of inflammation since ATX inhibition in an air pouch-induced murine model of inflammation ablates LPA production in the air pouch fluid (Gierse et al., 2010). ATX expression is up-regulated in response to a number of inflammatory stimuli, including TNF, LPS and bleomycin (Nakasaki et al., 2008; Li and Zhang, 2009; Nikitopoulou et al., 2012; Oikonomou et al., 2012).

1.4.3. LPA and its receptors in airway inflammation

Many in vitro studies have shown that LPA is able to regulate inflammation and LPA has been implicated in a number of inflammatory conditions, including rheumatoid arthritis, pruritus (itch), hepatitis and atherosclerosis (Watanabe et al., 2007; Nochi et al., 2008; Kremer et al., 2010; Zhou et al., 2011; Sevastou et al., 2013). Additionally, an in vivo study of LPA-induced inflammation using a murine air-pouch model showed that LPA increased leukocyte recruitment and cytokine secretion at the site of inflammation (Zhao et al., 2011c). In the airways and in cultures of airway cells, LPA has been shown to affect inflammation by several means; transcription factor activation, affecting interleukin expression, inducing expression of decoy receptors and affecting the activity of inflammatory cell types (Sevastou et al., 2013).

In cultured hASM and human lung epithelial cells, LPA activates a variety of transcription factors, including AP-1, NFκB, CREB, NFAT and SRE (Ediger et al., 2003; Zhao et al., 2005; Saatian et al., 2006). AP-1 and NFκB are known mediators of
inflammatory responses and are pivotal regulators in asthma (Barnes and Adcock, 1997; Nguyen et al., 2003). One well-established effect of LPA-induced AP-1 and NFκB activation is IL-8 production (Zhao et al., 2005; Saatian et al., 2006). IL-8 is a key mediator in the innate immune response and potent chemoattractant and activator of neutrophils (Pease and Sabroe, 2002). LPA-induced expression of IL-8 has been demonstrated in human airway epithelial cells and various cultured cell types (Zhao et al., 2006; Chiu et al., 2007; Chen et al., 2008). LPA-induced IL-8 expression is mediated through LPA1 and LPA3, but not LPA2 (Saatian et al., 2006). The involvement of other LPA receptors has not been investigated. IL-8 production may be a physiologically relevant function of LPA stimulation, since inhalation of LPA induced an increased neutrophil count in guinea pig BAL fluid (Hashimoto et al., 2003). Similarly, intratracheal administration of LPA elevated levels of the murine IL-8 homolog in BAL fluid and induced an increase in BAL fluid neutrophils in mice (Cummings et al., 2004). LPA is substantially less potent than LPC in this effect and it has been postulated that LPA acts therapeutically (Fan et al., 2008). In endothelial cells, LPA stimulates release of many pro-inflammatory mediators that could result in leukocyte recruitment; IL-8, MCP-1, pentraxin-3 and CXCL1 (Gustin et al., 2008; Shimada and Rajagopalan, 2010; Zhou et al., 2011; Mu et al., 2012).

LPA has also been shown to regulate T_{H}2 responses, which are more pertinent to atopic asthma. IL-13 is a key mediator of T_{H}2 inflammatory responses and is critical in the pathogenesis of inflammatory airways disease (Zhu et al., 1999; Ingram and Kraft, 2012). IL-13 exposure is sufficient to induce an asthma-like phenotype in mice and polymorphisms in the IL13 gene have been associated with asthma risk (Zhu et al., 1999; Bottema et al., 2010). The IL-13 pathway is being targeted for new asthma therapies (Corren et al., 2011; Ingram and Kraft, 2012). IL-13 mediates its effect by binding IL-13Rα1 and IL-4Rα (LaPorte et al., 2008). This activity is thought to be regulated by a decoy receptor IL-13Rα2, which binds IL-13 with a much higher affinity than IL-13Rα1 (Caput et al., 1996). A study using an ovalbumin-sensitised, induced asthma model in IL-13Rα2 knockout mice showed that IL-13Rα2 attenuates IL-13 effects in vivo, including inflammation, mucus metaplasia, TGF-β release and fibrosis (Zheng et al., 2008). LPA treatment of primary cell cultures of human bronchial epithelial cells induces IL-13Rα2 mRNA and protein expression and attenuates IL-13-induced activation of the transcription factor, STAT6. This LPA effect could be
inhibited by expression of catalytically inactive ATX or pre-treatment with PTx (Zhao et al., 2007). However, LPA has also been shown to induce IL-13 expression in T cells (Rubenfeld et al., 2006). LPA has also been shown to stimulate expression of secreted ST2 in cultured human bronchial epithelial cells (Zhao et al., 2012). IL-33 is a pro-inflammatory cytokine that activates T_{h2} responses via binding to the ST2 receptor and secreted ST2 acts a decoy receptor for IL-33 (Yagami et al., 2010).

In addition to its roles in regulating interleukins, LPA has been shown to affect expression of COX2 and release of PGE_2. LPA-induced COX2 expression has been demonstrated in cultured ovarian cancer cells, endometrial cells, colonic myofibroblasts and, of particular note here, human bronchial epithelial cells (He et al., 2008; Oyesanya et al., 2008; Woclawek-Potocka et al., 2009; Rodriguez Perez et al., 2011). In the airways COX2 expression is induced in response to allergen challenge in guinea pigs and PGE_2 is thought to have a protective effect since COX2 knockout mice display potentiated inflammation compared to wild-type animals when sensitised with ovalbumin as a model of atopic asthma (Oguma et al., 2002; Nakata et al., 2005). This correlates with a study of human volunteers with mild asthma which indicated that inhalation of PGE_2 prior to allergen challenge attenuated inflammation (Gauvreau et al., 1999).

Though many of the LPA effects discussed thus far have been anti-inflammatory, the demonstrated effects of LPA on leukocytes are often pro-inflammatory. Eosinophils express LPA_1 and LPA_3 at the mRNA level, and LPA stimulates activation of peripheral human eosinophils, as measured by eosinophil chemotaxis, production of reactive oxygen species and cell-adhesion proteins (Idzko et al., 2004). Mast cells isolated from human umbilical cord blood express LPA_1, LPA_2, LPA_3 and LPA_4 mRNA and including LPA in their culture medium accelerates their proliferation and maturation through an LPA_1/LPA_3-dependent mechanism (Bagga et al., 2004). A subsequent study showed that LPA treatment also induced mast cell secretion of pro-inflammatory cytokines via LPA_2 in a pathway that requires IL-4 (Lin and Boyce, 2005). These findings highlight the pleiotropic effects of LPA in cells expressing multiple LPA receptor subtypes, effects which are also modified by exposure to an inflammatory environment. In cultured T-lymphocytes, LPA treatment induces chemotactic responses, IL-2 generation, IL-13 secretion and prolongs cell survival, with
LPA\textsubscript{1} and LPA\textsubscript{2} differentially mediating responses (Goetzel et al., 1999; Zheng et al., 2000; Zheng et al., 2001; Rubenfeld et al., 2006). In neutrophils, LPA can stimulate cell migration, degranulation and further recruitment of neutrophils (Chettibi et al., 1994; Tou and Gill, 2005). In monocytes and macrophage cell-lines, LPA can induce IL-1 expression, production of reactive oxygen species and Ca\textsuperscript{2+} influx (Fueller et al., 2003; Chang et al., 2008).

In summary, LPA has been shown to regulate both pro- and anti-inflammatory effects and there is still much more to learn about the roles LPA has in regulating inflammation in the lung and how these roles are affected by the inflammatory environment of airways diseases such as asthma.

1.4.4. LPA and its receptors in airway remodelling

Many studies have pointed to a role for LPA in wound healing. LPA is produced by activated platelets (Eichholtz et al., 1993) and is increased at sites of injury and inflammation (Gierse et al., 2010). It can induce proliferation, contraction and migration in fibroblasts (Mio et al., 2002; Tager et al., 2008) and proliferation and migration of other structural cell-types, including smooth muscle cells and epithelial cells (Ceritis et al., 1997; Zhao et al., 2011). Furthermore, studies show that LPA can accelerate wound healing \textit{in vivo} (Sturm et al., 1999; Demoyer et al., 2000; Balazs et al., 2001). A clear role for LPA has already been demonstrated in pulmonary and renal fibrosis which can be described as conditions of aberrant and excessive wound healing (Pradere et al., 2008; Tager et al., 2008). Similarly, the remodelling that occurs in the airways is seen as a sort of excessive wound healing (Bousquet et al., 2000) and so interest in LPA as a potential mediator of remodelling has developed. LPA has also been linked to similar remodelling events in the vasculature that occur in atherosclerosis (Schober and Siess, 2012).

In hASM, human airway epithelial cells and human lung fibroblasts, LPA has been shown to induce proliferation and migration (Ediger and Toews, 2000; Tager et al., 2008; Zhao et al., 2011). In hASM, LPA-induced migration appears to require both G\textsubscript{q/11} involvement, while in lung epithelial cells PKC\textsuperscript{δ} is implicated (Hirshman and Emala, 1999; Zhao et al., 2011). In the case of LPA-induced proliferation of hASM cells, there is considerable cross-talk observed, with LPA
displaying a potent synergistic effect with EGF (Ediger and Toews, 2000). Increased hASM proliferation could contribute to the thickening of the smooth muscle layer observed in asthma. The importance of LPA-induced proliferation and migration in a relevant physiological setting and its ability to modify lung structure is demonstrated by its established role in pulmonary fibrosis (Tager et al., 2008).

The airway epithelium is an important structural component of the body's innate immune defences and there is evidence to show it is dysregulated in asthma, such that the epithelium is more susceptible to damage and less capable of effective repair (Knight and Holgate, 2003). A study of the effects of LPA on airway epithelial barrier integrity showed that LPA promotes accumulation of E-cadherin at cell-cell junctions and increased transepithelial electric resistance in vitro. In a murine model of lung damage, intratracheal injection of LPA reversed lipopolysaccharide-induced neutrophil infiltration, E-cadherin shedding and protein leak into BAL fluid (He et al., 2009). It has therefore been suggested that LPA mediates anti-inflammatory enhancement of epithelial barrier integrity, though this stands at odds with other reports that show that LPA exposure increases leukocyte infiltration in guinea pigs and mice (Hashimoto et al., 2001; Hashimoto et al., 2003; Cummings et al., 2004).

1.4.5. Effects of LPA and its receptors on contractility

In addition to the synthetic and remodelling functions already discussed, there is evidence to suggest that LPA can contribute to smooth muscle hypercontractility. One of the first studies to suggest a role for LPA in asthma concerned its effects on airway smooth muscle contractility; LPA treatment enhances the contractile response of isolated tracheal smooth muscle rings to methacholine, serotonin and substance P challenge without inducing a contraction when added alone. It also inhibits smooth muscle relaxation in response to the β-adrenoceptor agonist isoprenaline and the direct adenyl cyclase activator, forskolin (Toews et al., 1997). These observations suggest that LPA could contribute to a hypercontractile phenotype as seen in asthma. This theory is supported by in vivo studies in guinea pigs which showed that LPA inhalation induced hyperresponsiveness to acetylcholine by a mechanism involving Rho/ROCK (Hashimoto et al., 2001; Hashimoto et al., 2002).
LPA has also been shown to induce or augment contractile responses in isolated guinea pig ileum, rat colon, rat uterine smooth muscle, rat cardiac myocytes and collagen gels seeded with bovine tracheal smooth muscle cells or human lung fibroblasts (Tokumura et al., 1980; Tokumura et al., 1991; Mori and Tsushima, 2000; Mio et al., 2002; Cremers et al., 2003; Sakai et al., 2003). In several cases, this effect was PTx-sensitive or could be reversed by addition of cAMP-elevating agents, indicating Gα_{i/o} and cAMP involvement (Toews et al., 1997; Cremers et al., 2003; Sakai et al., 2003).

1.4.6. LPA and its receptors in models of asthma

Clearly, LPA is able to induce and modulate several of the cellular processes involved in asthma pathogenesis. However, LPA involvement in asthma is less well studied in in vivo models and humans. In human asthmatics, two studies by separate groups show that LPA levels in BAL fluid are increased by segmental allergen challenge (Georas et al., 2007; Park et al., 2013). The more recent of these analyses also showed an increase in ATX protein levels in BAL fluid from allergen challenged asthmatics (Park et al., 2013). Similarly, a study using a model wherein mice are sensitised with Schistosoma mansoni eggs, subsequent challenge with the sensitising agent induces an increase in LPA levels (Zhao et al., 2009).

The increased LPA levels observed may contribute to the lymphocyte infiltration observed in asthma, since an in vivo study in guinea-pigs showed that LPA exposure could significantly increase BAL fluid levels of eosinophils and neutrophils (Hashimoto et al., 2001). This infiltration was found to be mediated by a Rho/ROCK-dependent pathway (Hashimoto et al., 2003). Similar studies using mouse models showed that LPA challenge caused an increase in BAL fluid neutrophils, with one study linking neutrophil infiltration to IL-8 secretion (Cummings et al., 2004; Zhao et al., 2009). In one study, allergen-induced inflammation was significantly enhanced in LPA₂ knockout mice, pointing to an overall anti-inflammatory effect of this LPA receptor (Emo et al., 2012), however a recent analysis demonstrated that ATX or LPA₂ knockout significantly attenuated levels of Th2 cytokines and airway inflammation (Park et al., 2013).
Additionally, the gene for ATX was identified in a genome-wide linkage analysis and transcript profiling study as a gene associated with altered lung development, function and remodelling (Ganguly et al., 2007).

1.4.7. **Role of LPA and its receptors in pulmonary fibrosis**

In addition to a possible role for LPA in asthma, a link between LPA$_1$ receptor signalling and pulmonary fibrosis has been established in a study by Tager et al. (2008). This group reported that LPA levels were increased in BAL fluid from mice after challenge with bleomycin (an agent commonly used to create an animal model of idiopathic pulmonary fibrosis (IPF)). Mice lacking LPA$_1$ were significantly protected from bleomycin-induced fibrosis and consequent mortality. In IPF, excessive fibroblast accumulations and increased vascular permeability are observed in the injured lung, and deletion of the LPA$_1$ receptor was able to significantly attenuate these responses. Moreover, LPA levels in BAL fluid from patients with IPF were also increased compared to those obtained from healthy donors and pharmacological inhibition of the LPA$_1$ receptor was able to reduce chemotactic responses of fibroblasts to this fluid (Tager et al., 2008). Follow-up studies show that ATX up-regulation in fibrosis contributes to disease (Oikonomou et al., 2012). These are important findings since the same group had previously provided evidence to suggest that inhibiting fibroblast migration can attenuate pulmonary fibrosis (Tager et al., 2004). Some of these findings may also be of importance for other respiratory diseases, since airway narrowing and inflammation are also characteristics of asthma and chronic obstructive pulmonary disease (Barnes et al., 2006).

1.5. **Project aims**

As discussed here, there is a substantial body of evidence implicating LPA and LPA signalling in asthma pathogenesis, though there has been little opportunity to perform such studies in cells or tissues from human asthmatics. In this project I aim to characterise LPA receptor signalling in cultured human airway smooth muscle cells from control and clinically characterised asthmatic donors.

To better understand the impact of asthma on gene expression, particularly in the context of LPA signalling and metabolism, I will compare the expression of genes
involved in inflammation and various stages of LPA signalling in cultured hASM cells isolated from control and asthmatic individuals.

I will develop assays to characterise the activity of LPA receptor ligands at LPA receptors, including various LPA species and reported receptor agonists and antagonists. This will allow consolidation of data regarding these species, and may provide some useful tools to aid study of individual receptor subtypes.

While we know that LPA levels may be increased in airway inflammation (Georas et al., 2007), little is known about the signalling that occurs in hASM following this LPA stimulation and such events have never been studied in cells from asthmatic donors. Using cultured hASM cells from clinically characterised control and asthmatic donors, I will investigate the signalling events that occur downstream of LPA stimulation. Since the adenylyl cyclase/cAMP pathway is of particular pertinence and is an established effect of LPA stimulation (Ishii et al., 2000; Choi et al., 2010), initial study will focus on this pathway. LPA-induced migration of control and asthmatic cultured hASM cells will also be investigated.
CHAPTER 2. Materials and Methods
2.1. Materials

All LPAs and lipid ligands were purchased from Avanti Polar Lipids, Alabama, USA. See Table 2.1 and Table 2.2 for structures of lipids used. Cell culture media, foetal bovine serum (FBS), phosphate-buffered saline (PBS), fungizone, penicillin, streptomycin, cell dissociation buffer, trypsin/EDTA, non-essential amino acids, HEPES, sodium pyruvate, genetisin sulphate (G418), native Taq DNA polymerase, Express SYBR® GreenER™ qPCR SuperMix and Fluo-4-AM (fluor-4-acetoxyethyl ester) were from Invitrogen, California, USA. [²⁻³H]cAMP and [³⁵S]GTPγS, scintillation proximity assay (SPA) beads and Safe-Fluor scintillant were from Perkin Elmer, Massachusetts, USA. Calcium-4 assay kits were from Molecular Devices, California, USA. Bradford assay reagents were from Bio-Rad, California, USA. peqGOLD Total RNA kit and DNase I were from PEQLAB Biotechnologie GmbH, Erlangen, Germany. SuperScript® VILO™ cDNA Synthesis Kit from Life Technologies, California, USA. All TaqMan® reagents, assays and TLDA cards are from Applied Biosystems, California, USA. Custom primers were from Eurofins MWG Operon, Ebersberg, Germany. Plasmids containing rat and human LPA receptor CDS were kindly provided by Novartis. Antibodies were from Cell Signalling Technologies, Massachusetts, USA. DNA ladder and loading dye were from ThermoFisher Scientific, Massachusetts, USA. GelRed™ nucleic acid stain was from Biotium, California, USA. Transwell cell culture inserts were from BD Biosciences, New Jersey, USA. All other reagents and chemicals were from Sigma-Aldrich, Missouri, USA.

2.2. Cell culture methods

2.2.1. Culture of cell lines

RH7777 cells, either WT or expressing the LPA₁ receptor, were maintained in Dulbecco’s minimum essential medium (DMEM) containing 3.97 mM L-glutamine, 1 mM sodium pyruvate and 25 mM glucose, supplemented with 10 % FBS, 2.5 µg mL⁻¹ fungizone, 50 units mL⁻¹ penicillin and 50 µg mL⁻¹ streptomycin. For transfected cell-lines 500 µg mL⁻¹ G418 was also added for selection.
Table 2.1
Table showing chemical structures of LPA species used in the pharmacological profiling study.
<table>
<thead>
<tr>
<th>Lipid</th>
<th>Chemical Structure</th>
<th>Reported Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDP</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>Reported competitive inhibitor at LPA$_1$ and LPA$_3$ and agonist at LPA$_2$</td>
</tr>
<tr>
<td>N-P-Serine-PA</td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>Some controversy</td>
</tr>
<tr>
<td>N-P-Tyrosine-PA</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>Some controversy</td>
</tr>
<tr>
<td>VPC12249</td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td>Reported LPA$_1$ and LPA$_3$ selective antagonist</td>
</tr>
<tr>
<td>VPC 32179 (R)</td>
<td><img src="image5" alt="Chemical Structure" /></td>
<td>Reported LPA$_1$ and LPA$_3$ selective antagonist</td>
</tr>
<tr>
<td>VPC 32183 (S)</td>
<td><img src="image6" alt="Chemical Structure" /></td>
<td>Reported LPA$_1$ and LPA$_3$ selective antagonist</td>
</tr>
<tr>
<td>VPC 31143 (R)</td>
<td><img src="image7" alt="Chemical Structure" /></td>
<td>Reported LPA receptor agonist</td>
</tr>
<tr>
<td>VPC 31144 (S)</td>
<td><img src="image8" alt="Chemical Structure" /></td>
<td>Reported LPA receptor agonist</td>
</tr>
<tr>
<td>2(S)-OMPT</td>
<td><img src="image9" alt="Chemical Structure" /></td>
<td>Reported LPA$_3$ selective agonist</td>
</tr>
</tbody>
</table>

**Table 2.2**

Table showing chemical structures of lipid agonists and antagonists used in the pharmacological profiling study.
SW982 cells were maintained in DMEM containing 25 mM glucose and 1 mM sodium pyruvate, supplemented with 2 mM L-glutamine, 10 % FBS, 2.5 μg mL⁻¹ fungizone, 50 units mL⁻¹ penicillin, 50 μg mL⁻¹ streptomycin.

Chem-1 cells were maintained in DMEM containing 25 mM glucose and 3.97 mM L-glutamine, supplemented with 10 % heat inactivated FBS, non-essential amino acids, 10 mM HEPES, 50 units mL⁻¹ penicillin and 50 μg mL⁻¹ streptomycin. For transfected cell-lines 500 μg mL⁻¹ G418 was also added for selection. Chem-1 cell-lines stably transfected to express LPA₁ or LPA₃ were purchased from Millipore. Chem-1 cell lines stably transfected to express LPA₂ were developed by Novartis.

All cell types were maintained by incubation at 37 °C in a 5 % CO₂-air humidified atmosphere. When confluent, flasks of cells were passaged by washing twice with PBS (without Ca²⁺/Mg²⁺) and harvesting with 0.25 % (w/v) trypsin, 0.02 % (w/v) EDTA or cell dissociation buffer.

2.2.2. Isolation, establishment and culture of primary human airway smooth muscle cells

Human airway smooth muscle (hASM) cells were isolated by microscopic dissection from bronchial biopsies and surgical resections, with NHS Regional Ethics Committee approval. Explants were grown in 6-well culture plates with growth medium (DMEM containing 3.97 mM L-glutaMAX™, 1 mM sodium pyruvate and 25 mM glucose, supplemented with 10 % FBS, non-essential amino acids, 2.5 μg mL⁻¹ fungizone, 50 units mL⁻¹ penicillin and 50 μg mL⁻¹ streptomycin) at 37 °C in a 5 % CO₂-air humidified atmosphere for up to 1 month. Isolation and initial primary cell culture stages were performed by clinicians and researchers in Department of Respiratory Sciences, Glenfield Hospital. Once confluent, the hASM cells were passaged using 0.25 % (w/v) trypsin, 0.02 % (w/v) EDTA into 75 cm² culture flasks at a minimum density of 200,000 cells per flask; this was denoted passage 1 (P1). A smooth muscle phenotype was characterised by >90 % α-smooth muscle actin staining in flow cytometry experiments. Cells were used at a maximum passage of P4.
2.3. **RNA isolation, quantification and generation of cDNA samples**

Normal steps to prevent RNAsé contamination were taken whenever working with RNA including using disposable gloves, autoclaved tubes and nuclease-free pipette tips and water.

2.3.1. **RNA isolation and quantification**

A monolayer of cells in a 10 cm culture dish was grown to near confluency. Total RNA was isolated using a peqGOLD Total RNA Kit according to manufacturer's instructions. Briefly, cells were washed twice with PBS and lysed with the provided lysis buffer. The lysate was transferred to a DNA removing micro-column in a collection tube and centrifuged at 12,000 g for 1 min. The flow-through was transferred to a new tube and 70 % ethanol was added at 1:1 (v/v). This mixture was loaded onto a PerfectBind RNA isolating silica micro-column and centrifuged at 10,000 g for 1 min. The column, with RNA bound, was then washed once with the provided wash buffer and centrifuged at 10,000 g for 15 sec. DNAse 1 reaction mix was then added directly to the column filter and incubated for 15 min at 26 °C to remove any remaining contaminating DNA. The column was then washed three further times with the provided wash buffers according to the manufacturer's instructions, centrifuging between washes. The column was then dried by centrifuging at 10,000 g for 1 min to remove any ethanol. The RNA on the column was then eluted into a fresh microfuge tube by adding 50 µL water, incubating at room temperature for 5 min and centrifuging at 5,000 g for 1 min. RNA was stored at -80 °C.

RNA was quantified using an Infinite® 200 PRO Nano Quant spectrophotometer from Tecan (Männedorf, Switzerland). Absorbance at 260 nM of a 1.5 µL sample of RNA was measured in a Nano Quant plate.

2.3.2. **Reverse transcription to generate cDNA**

0.5 - 2 µg RNA underwent reverse transcription using the SuperScript® VILO™ cDNA Synthesis Kit according to the manufacturer's instructions. Briefly, a reaction mixture containing 1 x VILO™ reaction buffer, 1 x SuperScript® reverse transcriptase enzyme, RNA sample and nuclease free water to volume was prepared; a -RT control mixture
was also prepared in parallel for each sample which contained water in place of enzyme. Note that the reaction mixture contains both oligo-d(T)_{16} and random hexamers for priming. Reaction mixtures were gently mixed, incubated at 25 °C for 10 min, then 42 °C for 60 min. The reaction was terminated by incubation at 85 °C for 5 min and the cDNA samples were stored at -20 °C. Stated cDNA concentrations are inferred from the determined RNA concentrations, assuming 100% reaction efficiency.

2.4. **PCR methods**

2.4.1. **Design of primers for rat and human LPA receptors**

Coding sequences (CDS) for the rat and human LPA receptors 1-6 were identified using the National Centre for Biotechnology Information (NCBI) gene database. Primers were designed within the CDS using NCBI's Primer-BLAST which uses Primer3 (Rozen and Skaletsky, 2000) algorithms to design primers and submits the resulting primer sequences to BLAST search against selected databases to ensure specificity for intended targets. Here, the primer sequences were searched against *Homo sapiens* and *Rattus norvegicus* databases to ensure species specificity. The following restraints were applied for primer design: a primer length of 18 - 24 bp with an optimum length of 20 bp, a product size of between 100 and 800 bp, a T_{m} of between 57 and 63 °C with an optimum T_{m} of 60 °C, and a GC content of between 40 and 60%. Where feasible, a GC clamp was also included. The maximum allowed self-complementarity score was set to 3 overall and 3 at the 3' end (Rozen and Skaletsky, 2000). The minimum desirable mismatches for other targets in the selected database was set to 4, including 2 in the final 5 bp. For details of primer sequences please see Table 2.3.

2.4.2. **RT-PCR and agarose gel electrophoresis**

PCR reaction mixtures were assembled in 0.2 mL PCR tubes according to the composition described in Table 2.4. Where possible, master mixes were prepared for multiple reactions in order to minimise variability in reaction composition.

PCR was performed according to the Taq polymerase manufacturer's recommendations using a thermal cycler (Applied Biosystems, GeneAmp). General program parameters were as follows:
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>NCBI Accession Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RN18S1</strong></td>
<td>NR_003286</td>
<td>891 GTTGGTTTTTCGGAACTGAGG</td>
<td>1090 GCATCGTTTTATGGTCGGAAC</td>
</tr>
<tr>
<td><strong>hLPAR1</strong></td>
<td>NM_001401.3</td>
<td>834 GCCGGTAGTGGTGGTGTCTTGTG</td>
<td>1403 ATGGTGTGTTGAGGGAGGAGGAAGC</td>
</tr>
<tr>
<td><strong>rLpar1</strong></td>
<td>NM_053936.3</td>
<td>316 CTTGCGCACTACTGCTGCTGTTTC</td>
<td>963 GCAGACAAATAAAGGCACCCACGAC</td>
</tr>
<tr>
<td><strong>hLPAR2</strong></td>
<td>NM_004720.5</td>
<td>157 GTGCTACTACAAGAGACCATCGG</td>
<td>442 AAGTGGAAGTCCGGCTGTGCG</td>
</tr>
<tr>
<td><strong>rLpar2</strong></td>
<td>NM_001109109.1</td>
<td>393 CTCTCCATCAAAGGCTGGTTCCTG</td>
<td>567 CCACCCACACTCCACGATGA</td>
</tr>
<tr>
<td><strong>hLPAR3</strong></td>
<td>NM_012152.2</td>
<td>436 GTGCCGGATTTCCTCCTGCTGGA</td>
<td>575 AAGTGGTGAAGGGAAGCACTGCAAG</td>
</tr>
<tr>
<td><strong>rLpar3</strong></td>
<td>NM_023969.1</td>
<td>84 TGTGCCCTTGGAGCATTTCTCTGCG</td>
<td>218 CAAAGAAATCCGGCCACCGCAAG</td>
</tr>
<tr>
<td><strong>hLPAR4</strong></td>
<td>NM_005296.2</td>
<td>870TGCCATTGTGTGTGCCTGCTGTG</td>
<td>1229 ACCAGGGCATACAAGAGAGGAAC</td>
</tr>
<tr>
<td><strong>rLpar4</strong></td>
<td>NM_001106940.1</td>
<td>723 AGTGCTTTCCTGTTTGTCTTCTGG</td>
<td>1112 AGTGTTGTTGCCATTTGTGAC</td>
</tr>
<tr>
<td><strong>hLPAR5</strong></td>
<td>NM_001142961.1</td>
<td>665 TGCCCCTTCGGTCTCTCTACTACG</td>
<td>902 ACGGCAAACACCAGGATGAC</td>
</tr>
<tr>
<td><strong>rLpar5</strong></td>
<td>XM_001063300.2</td>
<td>776 TTCTGCTGTTGCTTCTGC</td>
<td>1053 TTCTGAGGGTGTTTGCGTAC</td>
</tr>
<tr>
<td><strong>hLPAR6</strong></td>
<td>NM_005767.5</td>
<td>1750 TACATCTGGCTCCTAAGAGTCGCA</td>
<td>2098 TGAGTACCTGAACAAAAACCGC</td>
</tr>
<tr>
<td><strong>rLpar6</strong></td>
<td>NM_001045843.1</td>
<td>623 ACAGGCTCCTCGCCTATCGA</td>
<td>1056 CGCACTTCTCCCATGCGT</td>
</tr>
</tbody>
</table>

**Table 2.3**

Table of sequences and details of primers used for RT-PCR
<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
<th>µL in 50 µL reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x PCR buffer</td>
<td>1 x</td>
<td>5</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>1.5 mM</td>
<td>1.5</td>
</tr>
<tr>
<td>10 µM each dNTP mix</td>
<td>0.2 µM</td>
<td>1</td>
</tr>
<tr>
<td>10 µM each primer mix</td>
<td>0.2 µM</td>
<td>1</td>
</tr>
<tr>
<td>5 U µL⁻¹ Taq polymerase</td>
<td>1 U per reaction</td>
<td>0.2</td>
</tr>
<tr>
<td>DNA template</td>
<td>50 ng cDNA or 1 ng plasmid</td>
<td>Variable</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>To volume</td>
<td>To 50 µL</td>
</tr>
</tbody>
</table>

**Table 2.4**

Table illustrating the general recipe used for RT-PCR experiments. *Taq* polymerase is native, unmodified enzyme isolated from *Thermus aquaticus*. PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl) and MgCl₂ were provided by manufacturer with the polymerase.
94 °C 3 min Initial denaturation of template
94 °C 45 s Denaturation
x °C 30 s Annealing x 30 cycles
72 °C 1 min 30 s Extension
72 °C 10 min Final extension

The annealing temperature used was 5 °C below the lowest primer T<sub>m</sub>. Please see Table 2.3 for primer T<sub>m</sub> values.

PCR products were added to 6 x DNA loading dye (10 mM Tris-HCl (pH 7.6), 0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 60 % glycerol and 60 mM EDTA) to give a 1 x final concentration, then visualised by agarose gel electrophoresis. Agarose was dissolved in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) and 1:10,000 GelRed™ nucleic acid stain added to make gels. Agarose concentration was 1.5 % where the expected product size is ≤ 150 bp and 2 % for smaller products. 12 µL PCR product and loading dye mixture was loaded into each well and gels were run in TAE buffer at 100 V for approximately 70 min. 0.5 µg GeneRuler™ low-range DNA ladder was used as a size marker. Gels were viewed and imaged under UV light.

2.4.3. **RT-qPCR using primers for 18S to assess genomic contamination of cDNA**

Reaction mixes were prepared in 0.2 µL PCR tubes for the RT+ and RT- preparations of all cDNAs used in duplicate with the following final composition; 1 x Express SYBR® GreenER™ qPCR SuperMix, 0.2 µM forward primer, 0.2 µM reverse primer, 0.5 ng cDNA template and PCR grade water to 20 µL volume. Note that the Express SYBR® GreenER™ qPCR SuperMix contains Platinum® Taq DNA polymerase, SYBR® GreenER™ fluorescent dye, MgCl<sub>2</sub>, heat-labile uracil DNA glycosylase (UDG), dNTPs and appropriate buffer. Forward and reverse primers amplified a 200 bp product. Please see Table 2.3 for primer sequence and details.

PCRs were run using a Roche LightCycler® with the following program parameters:

<table>
<thead>
<tr>
<th>Temp.</th>
<th>Time</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 °C</td>
<td>2 min</td>
<td>UDG incubation</td>
</tr>
<tr>
<td>95 °C</td>
<td>5 min</td>
<td>Initial template denaturation and enzyme hot-start</td>
</tr>
</tbody>
</table>
The threshold was set to 0.005 arbitrary fluorescence units for analysis.

2.4.4. **RT-qPCR using TaqMan® low density arrays**

TaqMan® low density arrays (TLDAs) pre-loaded with desiccated TaqMan® assay components (primers and hydrolysis probes) were used to perform high-throughput RT-qPCR. Please see Figure 2.1 for images of the array cards and Table 2.5 and Table 2.6 for a list of the gene targets on the TLDA cards used. The two card-set ups carried assay components for targets related to inflammation and remodelling or LPA metabolism and signalling. For ease, they will be referred to as the inflammation TLDA and LPA TLDA respectively.

Reaction mixtures were prepared for each cDNA sample as follows, 60 µL 2 x TaqMan® fast universal PCR master mix, 100 ng cDNA and PCR grade water to 120 µL volume. 100 µL of the reaction mixture was loaded into the loading chambers of the array card and the assay wells were filled by centrifuging the cards in specialised centrifuge buckets (Applied Biosystems) for 2 x 1 min at 331 g. The card is then sealed and the loading chambers removed with scissors. The card is then subjected to thermocycling in an Applied Biosystems 7900HT Fast Real-Time PCR machine.

The data were analysed by the comparative $C_t$ method which assumes that the efficiency of reaction for the various probes used is equal (Livak and Schmittgen, 2001).

2.4.5. **RT-qPCR using primer and hydrolysis probe sets**

RT-qPCR was performed using primer and hydrolysis probe sets and the Applied Biosystem 7500 Fast PCR system and were executed according to the manufacturer's instructions and as described here. The PCR reaction mixtures were assembled as follows; 10 µL 2 x TaqMan® fast universal PCR master mix, 1 µL 20 x assay mix, required amount of cDNA template and PCR grade water to 20 µL volume. Note that the master mix contains hot-start AmpliTaq® fast DNA polymerase, dNTPs and appropriate enzyme buffer. TaqMan® assay mix contains primers and target-specific
Figure 2.1

A, photograph of a TaqMan® low density array loaded with blue dye, showing the even loading of the assay wells after loading and centrifugation. B, a schematic of a TaqMan® low density array illustrating the narrow channels through which the assay wells are loaded by centrifugation.
Table 2.5
Table showing gene and gene product names of the targets assayed on the inflammation TLDA.
<table>
<thead>
<tr>
<th>Gene/Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPAR1</td>
<td>LPA₁ receptor</td>
</tr>
<tr>
<td>LPAR2</td>
<td>LPA₂ receptor</td>
</tr>
<tr>
<td>LPAR3</td>
<td>LPA₃ receptor</td>
</tr>
<tr>
<td>LPAR4</td>
<td>LPA₄ receptor</td>
</tr>
<tr>
<td>LPAR5</td>
<td>LPA₅ receptor</td>
</tr>
<tr>
<td>LPAR6</td>
<td>LPA₆ receptor</td>
</tr>
<tr>
<td>ENPP2</td>
<td>Autotaxin</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin-cholesterol acyltransferase</td>
</tr>
<tr>
<td>LIPH</td>
<td>Lipase, member H (mPA-PLA1)</td>
</tr>
<tr>
<td>LPPI</td>
<td>Lipase, member I (mPA-PLA1beta)</td>
</tr>
<tr>
<td>PLA1A</td>
<td>Phospholipase A1A</td>
</tr>
<tr>
<td>PLA2G2A</td>
<td>Secretory phospholipase A2</td>
</tr>
<tr>
<td>PLA2G4A</td>
<td>Cytosolic phospholipase A2</td>
</tr>
<tr>
<td>PPAP2A</td>
<td>Lipid phosphate phosphohydrolase 1</td>
</tr>
<tr>
<td>PPAP2B</td>
<td>Lipid phosphate phosphohydrolase 3</td>
</tr>
<tr>
<td>PPAP2C</td>
<td>Lipid phosphate phosphohydrolase 2</td>
</tr>
<tr>
<td>GNAS</td>
<td>Gαs</td>
</tr>
<tr>
<td>GNAI3</td>
<td>Gαi3</td>
</tr>
<tr>
<td>GNAI2</td>
<td>Gαi2</td>
</tr>
<tr>
<td>GNAI1</td>
<td>Gαi1</td>
</tr>
<tr>
<td>ADCY1</td>
<td>Adenyl cyclase 1</td>
</tr>
<tr>
<td>ADCY2</td>
<td>Adenyl cyclase 2</td>
</tr>
<tr>
<td>ADCY3</td>
<td>Adenyl cyclase 3</td>
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<td>Adenyl cyclase 6</td>
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<tr>
<td>ADCY7</td>
<td>Adenyl cyclase 7</td>
</tr>
<tr>
<td>ADCY8</td>
<td>Adenyl cyclase 8</td>
</tr>
<tr>
<td>ACTB</td>
<td>B-actin</td>
</tr>
<tr>
<td>B2M</td>
<td>β2 microglobulin</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HMBS</td>
<td>Hydroxymethylbilane synthase</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
</tr>
<tr>
<td>PGK1</td>
<td>Phosphoglycerate kinase 1</td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidylprolyl isomerase A</td>
</tr>
<tr>
<td>TFRC</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>PTGIR</td>
<td>Prostaglandin I2 receptor</td>
</tr>
<tr>
<td>PDE4A</td>
<td>Phosphodiesterase 4A</td>
</tr>
<tr>
<td>PDE4B</td>
<td>Phosphodiesterase 4B</td>
</tr>
<tr>
<td>RAPGEF3</td>
<td>RAP guanine nucleotide exchange factor 3</td>
</tr>
<tr>
<td>RAPGEF4</td>
<td>RAP guanine nucleotide exchange factor 4</td>
</tr>
<tr>
<td>RXFP2</td>
<td>Relaxin/insulin-like family peptide receptor 2</td>
</tr>
<tr>
<td>RXFP1</td>
<td>Relaxin/insulin-like family peptide receptor 1</td>
</tr>
<tr>
<td>SPHK1</td>
<td>Sphingosine kinase 1</td>
</tr>
<tr>
<td>SPHK2</td>
<td>Sphingosine kinase 2</td>
</tr>
</tbody>
</table>

**Table 2.6**

Table showing gene and gene product names of the targets assayed on the LPA TLDA.
hydrolysis probe. For experiments, 20 ng cDNA per reaction was used, while the amount of cDNA was varied for generation of standard curves. Master mixes were prepared where appropriate in order to minimise variability in reaction composition. Reactions were prepared in triplicate and loaded onto 96-well PCR reaction plates and sealed using a film specialised for optical clarity (Applied Biosystems). Reactions were run on Applied Biosystems Fast 7500 real-time PCR machine using the following program parameters:

\[
\begin{align*}
95^\circ C & \quad 20 \text{ s} & \text{Template denaturation and enzyme initialisation} \\
95^\circ C & \quad 3 \text{ s} & \text{Denaturation} \\
60^\circ C & \quad 30 \text{ s} & \text{Annealing and extension}
\end{align*}
\]

x 40 cycles

Standard curves for quantification were generated for each primer and hydrolysis probe set by determining $C_t$ values using a range of cDNA quantities; a blend of hASM cDNAs was used as template. The template was diluted 2-fold several times to generate a standard curve with the following amounts of total cDNA per reaction- 40, 20, 10, 5, 2.5, 1.25, 0.625 and 0 ng. Again, reactions were performed in triplicate for each primer and probe set. The standard curve was formed by plotting mean observed $C_t$ values against total cDNA per reaction. Relative quantities of unknown in samples were then calculated from the curve. For a list of the gene targets investigated, please see Table 2.7.

2.5. **Membrane preparation and total [\textsuperscript{35}S]GTP\gamma S binding assay**

RH7777 cells were grown to confluency; the growth medium was removed and cells were washed with warm HBS (10 mM HEPES, 0.9 % NaCl, pH 7.4). Cells were lifted with ice-cold harvesting buffer (10 mM HEPES, 0.9 % NaCl, 0.2 % EDTA, pH 7.4) and centrifuged at 500 g for 5 min at 4 °C. The supernatant was discarded and the pellet re-suspended in ice-cold solubilisation buffer (10 mM HEPES, 10 mM EDTA, pH 7.4), before homogenising (on ice) using an electrical homogeniser for four bursts of five seconds. The homogenate was centrifuged at 45,000 g for 30 min at 4 °C. The supernatant was discarded and the pellet was again re-suspended and homogenised in solubilisation buffer as described. The supernatant was discarded and the pellet re-suspended in ice cold re-suspension buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4).
<table>
<thead>
<tr>
<th>Gene target</th>
<th>Gene product</th>
<th>TaqMan® assay ID number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Hs_02758991_g1</td>
</tr>
<tr>
<td>SOCS1</td>
<td>suppressor of cytokine signaling 1</td>
<td>Hs_00705164_s1</td>
</tr>
<tr>
<td>IL1A</td>
<td>interleukin 1, alpha</td>
<td>Hs_00174092_m1</td>
</tr>
<tr>
<td>MIF</td>
<td>macrophage migration inhibitory factor</td>
<td>Hs_00236988_g1</td>
</tr>
<tr>
<td>CCL11</td>
<td>chemokine (C-C motif) ligand 11</td>
<td>Hs_00237013_m1</td>
</tr>
<tr>
<td>LIPH</td>
<td>lipase, member H</td>
<td>Hs_00975890_m1</td>
</tr>
<tr>
<td>PPAP2C</td>
<td>phosphatidic acid phosphatase type 2C, LPP2</td>
<td>Hs_00186575_m1</td>
</tr>
<tr>
<td>PDE4B</td>
<td>phosphodiesterase 4B, cAMP-specific</td>
<td>Hs_00963643_m1</td>
</tr>
<tr>
<td>PDE4D</td>
<td>phosphodiesterase 4D, cAMP-specific</td>
<td>Hs_01579625_m1</td>
</tr>
<tr>
<td>SPHK2</td>
<td>sphingosine kinase 2</td>
<td>Hs_00219999_m1</td>
</tr>
<tr>
<td>ADCY8</td>
<td>adenylate cyclase 8</td>
<td>Hs_00181588_m1</td>
</tr>
<tr>
<td>RAPGEF3</td>
<td>Rap guanine nucleotide exchange factor (GEF) 3</td>
<td>Hs_00183449_m1</td>
</tr>
<tr>
<td>GNAS</td>
<td>adenylate cyclase-stimulating G alpha protein</td>
<td>Hs00255603_m1</td>
</tr>
</tbody>
</table>

**Table 2.7**

Table showing the TaqMan® primer and probe assay numbers used for RT-qPCR and their corresponding human gene targets.
Protein concentration was determined using a Bradford protein assay according to manufacturer’s instructions and adjusted if required with re-suspension buffer. Aliquots were prepared and snap frozen before being stored at -80 °C. Aliquots were quickly defrosted at 30 °C and re-homogenised using a gauge 25 needle and syringe immediately before use.

RH7777 membranes (20 µg per well) were incubated with agonist (or buffer for basal), 3 µM GDP and 0.5 mg SPA beads in [35S]GTPγS binding assay buffer (10 mM HEPES, 100 mM NaCl, and 10 mM MgCl2, pH 7.4) for 10 min at room temperature, before addition of 300 pM [35S]GTPγS and incubation for a further 30 min. Quoted concentrations are final and the final volume was 250 µL in a 96-well plate. Plates were then centrifuged at 3000 r.p.m. for 3 min and read on a Topcount scintillation counter (Perkin Elmer, USA). Data are expressed as c.p.m or c.p.m. minus basal and expressed as a percentage of the response observed from stimulation with 1 µM LPA on the same plate. Note that conditions are as described above except where parameters were altered for optimisation as described in Results.

2.6. Measurement of changes in [Ca2+]i in a cell population using a Hamamatsu FDSS

Cells were seeded onto black-walled, clear-bottomed 96-well plates and grown for 24 hours at 37 °C in a humidified atmosphere + 5 % CO2. SW982 cells were seeded at 7,500 cells per well whereas Chem-1 cells were seeded at a density of 12,000 cells per well. Cells were serum-starved for 6 hours before experimentation by washing cells into serum-free media. Cells were then loaded with Calcium-4 no-wash Ca2+ dye for 1 hour at 37 °C. Desired agonist additions were performed by the FDSS and changes in fluorescence intensity for each well was recorded every 1 s for the first 40 s, then every 6 s for a further 19 readings, adding compounds at 11 s. The peak height (i.e. maximum minus minimum observed response) was recorded for each well.
2.7. cAMP measurement

2.7.1. Cell stimulations and sample preparation

Cells were seeded onto 24-well plates, grown to confluency and serum starved for 24 hours before experimentation. Medium was removed, cells washed twice with warm KHB (Krebs-Henseleit buffer - 118 mM NaCl, 8.5 mM HEPES, 4.7 mM KCl, 4 mM NaHCO$_3$, 1.3 mM CaCl$_2$, 1.2 mM MgSO$_4$.7 H$_2$O, 1.2 mM KH$_2$PO$_4$, and 11.7 mM glucose, pH 7.4) and KHB replaced with 400 µL KHB containing 500 µM isomethyl 1-butyl xanthine (IBMX). Cells were incubated at 37 °C for 20 min before addition of agonist (or KHB for basal). To terminate the assay, each well was aspirated, 400 µL ice-cold trichloroacetic acid (TCA, 0.5 M) added and plates placed on ice for 20 min. Samples were removed from each well to a fresh 1.5 mL microfuge tube and mixed with 50 µL EDTA (10 mM, pH 7.0) and 500 µL of a 1:1 (v/v) mixture of tri-n-octylamine and 1,1,2-trichlorofluoroethane. Several tubes of blank buffer were also prepared (containing 400 µL TCA (0.5 M), 50 µL EDTA (10 mM, pH 7.0) and 500 µL of oil mix). Samples and blank buffers were vortex-mixed and left for 15 min. Following this, they were centrifuged at 14,000 r.p.m. for 2 min and 200 µL of the upper aqueous phase was transferred into fresh microfuge tubes containing 50 µL NaHCO$_3$ (62.5 mM). Blank buffer samples were pooled.

2.7.2. cAMP assay

Known concentrations of cAMP were prepared from a 5 µM stock in blank buffer to generate a standard curve with concentrations of 10, 8, 6, 4, 2, 1, 0.5, 0.25 and 0.125 pmol in 50 µL. Blank buffer alone was used to determine total binding and the 5 µM cAMP was used to determine non-specific binding. 100 µL [${}^3$H]cAMP (approx. 75,000 d.p.m.) made up in Tris/EDTA buffer (50 mM TrisHCl, 4 mM EDTA, pH 7.4) was added to 50 µL of each standard curve cAMP concentration (in duplicate) and all cell-derived samples. 150 µL cAMP binding protein was added and tubes were vortex-mixed and left for at least 90 min to equilibrate. Unbound cAMP was removed by incubating with ice-cold charcoal solution (Tris/EDTA buffer, 0.2 % BSA, 0.5 % charcoal) for 10 min, vortex mixing and centrifugation at 14,000 r.p.m. A 400 µL sample of the supernatant was taken and mixed with 3.4 mL SafeFluor scintillation fluid. Radioactivity was determined by liquid scintillation counting.
2.7.3. **Lowry protein assay**

The remaining cell monolayers in the 24-well culture plates were solubilised by addition of 500 µL 0.1 M NaOH and protein content was assayed by the Lowry method. A BSA standard curve was prepared in 0.1 M NaOH vehicle with the following values: 0, 40, 80, 120, 160, 200 and 250 µg. Samples and standards were then incubated with 1 mL Lowry assay mix (a 100:1:1 mixture of 2 % Na₂CO₃, 0.4 % NaOH:1 % CuSO₄:2 % K/Na tartrate) for 10 min at room temperature. Three-fold diluted Folin-Ciocalteu reagent (100 µL) was then added and incubated for 15 min at room temperature. Absorbance at 750 nm was measured with a spectrophotometer.

2.7.4. **Data analysis**

Protein content in assay plates and cAMP content in samples were determined using the standard curves generated. Data are expressed as pmol cAMP per mg protein.

2.8. **Measurement of cell migration using a modified Boyden chamber Transwell assay**

A sub-confluent monolayer of cells in culture flasks was washed in serum-free media twice and incubated in serum-free media for 24 hours. A 24-well culture plate was prepared containing 500 µL agonists and antagonists at the desired final concentration and transwell filters were placed in the wells see Figure 2.2 for diagrams of the experimental set up. Cells were then washed twice with PBS (without Ca²⁺ or Mg²⁺) and harvested with trypsin/EDTA before being centrifuged at 390 g for 3 min. Cells were then resuspended in fresh serum-free media and counted using a haemocytometer, then resuspended to a concentration of 50,000 cells per 150 µL serum free media. 150 µL cell suspension was added to each transwell filter. Where antagonist incubations were performed, antagonist was also added to the cell suspension at the desired final concentration for 15 min before transfer to agonist containing wells. The plates were then incubated at 37 °C in a humidified atmosphere with 5 % CO₂ for 6 hours to allow cell migration.

Following incubation, cells in the transwell filter were removed using a cotton bud and the cells which had migrated to the underside of the filter were fixed and stained by the following method:
Figure 2.2

A, diagram of the transwell cell culture inserts used in migration assays showing the membrane with 8 µm gauge pores through which cells migrate and how they fit into standard cell culture plates. Note that while a 6 well set up is shown, 24 well plates and inserts were used throughout this work. Images adapted from manufacturer's promotional literature. B, a cartoon illustrating the experimental set up for migration assays.
- 3 x washes in PBS
- fixation in 100 % methanol for 20 min at -20 °C
- 3 x washes in PBS
- staining in 0.05 % propidium iodide for 15 min at room temperature
- 3 x washes in PBS

Filters were then removed using a scalpel and mounted onto microscope slides using fluorescent mounting media.

The number of migrated cells was then counted in three fields of view at 20 x magnification by fluorescence microscopy and a mean value was taken.

2.9. **ERK phosphorylation assay using Western blotting**

2.9.1. **Cell stimulation and lysis**

Cells were maintained at 37 °C in a humidified atmosphere with 5 % CO₂ as far as was possible until lysis. Monolayers of cells were grown to 90 % confluency in 6 cm culture dishes and the media was replaced with serum free media with two washes with serum free media and incubated for 24 hours. The serum free media was then replaced with KHB and cells were then equilibrated for 1 hour before stimulation with agonists for 10 min. Where used, antagonists were added 5 min prior to agonist stimulation. Stimulations were stopped by transferring dishes to ice, removing KHB, adding 150 µL lysis buffer (see Table 2.8 for lysis buffer composition) and scraping cells from the dish. The lysate was transferred to a microfuge tube on ice and vigorously vortex-mixed to aid lysis, then centrifuged at 14,000 r.p.m for 10 min at 4 °C. 120 µL supernatant was removed to a new tube and the lysate was stored at -80 °C until use.

2.9.2. **Bradford protein assay and sample preparation**

The protein concentration in samples was determined by Bradford assay. 1 mL 1:5 diluted Bradford reagent was added to 10 µL sample or BSA reference in a cuvette and incubated at room temperature for 5 min. The absorbance at 595 nm was then read. A BSA standard curve ranging from 0 - 20 µg was used to determine the protein concentration in samples. Protein concentrations were then altered to match that of the
<table>
<thead>
<tr>
<th>Buffer component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Glycerophosphate, pH 7.4</td>
<td>10 mM</td>
</tr>
<tr>
<td>EDTA, pH 8.0</td>
<td>1 mM</td>
</tr>
<tr>
<td>EGTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>Tris-HCl, pH 7.5</td>
<td>50 mM</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>1 mM</td>
</tr>
<tr>
<td>Sodium orthovanadate</td>
<td>1 mM</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>1.5 µM</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>2.3 µM</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1 %</td>
</tr>
<tr>
<td>Sodium Fluoride</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

**Table 2.8**

Table showing the composition of the lysis buffer used in ERK phosphorylation Western blot studies.
sample with the lowest concentration by adding the appropriate volume of lysis buffer. 4x sample buffer (see Table 2.9 for sample buffer composition) was then added to the sample to yield a 1x final concentration and the samples were mixed and denatured by boiling at 100 °C for 3 min.

2.9.3. **Western blotting**

20 µg sample was loaded onto 10% polyacrylamide gels and run at 180 V for approximately 90 min to allow protein separation (see Table 2.9 for gel and running buffer composition). Proteins were transferred from the gels to PVDF membranes using a semi-dry transfer cell (Bio-Rad) running at 15 V for 35 min. Non-specific binding to the PVDF membrane was blocked by incubation with TBS-T + 5% fat-free milk powder at room temperature for 1 hour. Excess milk was then removed by washing the membranes for 10 min three times with TBS-T at room temperature before overnight incubation of the membranes with primary antibody against total or phosphorylated ERK at 4 °C. The membranes were then washed for 10 min three times with TBS-T at room temperature before 1 hour's incubation with anti-rabbit HRP-conjugated secondary antibody. The membranes were then washed for 10 min three times with TBS-T at room temperature before being visualised by incubation with ECL reagent and exposure to hyperfilm.

2.10. **Data analysis**

Concentration-response curves were analyzed by non-linear regression using GraphPad Prism 5.0 software (San Diego, CA). For statistical tests, where only two datasets were being compared an unpaired Student’s t-test was used where \( p < 0.05 \) was deemed statistically significant. Where greater than two datasets were compared, one- or two-way analysis of variance (ANOVA) tests were used with \( p < 0.05 \) being accepted as significantly different. ANOVA tests were followed with the Bonferroni’s *post-hoc* test. All statistical tests were performed using GraphPad Prism 5.0 software unless otherwise indicated.
<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Buffer</td>
<td>62.5 mM tris (pH 6.8), 1 % SDS, 0.001 % bromophenol blue, 10 % glycerol, 2.5 % β-mercaptoethanol</td>
</tr>
<tr>
<td>10 % polyacrylamide gels</td>
<td>Running gel: 10 % acrylamide, 375 mM tris (pH 8.8), 0.1 % SDS, 0.1 % APS, 0.0004 % TEMED</td>
</tr>
<tr>
<td></td>
<td>Stacking gel: 5 % acrylamide, 125 mM tris (pH 6.8), 0.1 % SDS, 0.1 % APS, 0.001 % TEMED</td>
</tr>
<tr>
<td>Running buffer</td>
<td>25 mM tris, 192 mM glycine, 0.1 % SDS</td>
</tr>
<tr>
<td>TBS-T</td>
<td>20 mM tris, 137 mM NaCl, 0.1 % Tween® 20, pH 7.6</td>
</tr>
<tr>
<td>Transfer buffer</td>
<td>25 mM tris, 192 mM glycine, 0.02 % SDS, 20 % methanol</td>
</tr>
</tbody>
</table>

**Table 2.9**

Table showing the composition of solutions used in ERK phosphorylation Western blot studies.
CHAPTER 3. Investigation of transcriptional changes in cultured human airway smooth muscle cells from control and asthmatic donors
3.1. **Introduction**

There are extensive changes to lung physiology during asthma pathogenesis and changes at the fundamental level of transcription can occur. The aim for this aspect of the project was to determine if there are any transcriptional changes associated with asthma which are retained in the practised culture conditions of hASM cells from clinically characterised control and asthmatic donors. To this end, a broad range of gene targets related to asthma, inflammation and airways remodelling, as well as LPA metabolism and signalling, were selected. Expression of these genes was investigated using a high-throughput means of semi-quantitative RT-PCR. This method uses a microfluidic card which is loaded with validated primers and hydrolysis probes for each gene target to enable investigation of up to 384 genes of interest on a single card. While the scope of this method is large, it is not fully quantitative so it was used as a broad screen to identify any gene targets of potential interest which would then be followed up with a more intensive, quantitative study using individual primer and hydrolysis probe sets and the standard curve method for quantification.

3.1.1. **TLDA card composition**

The TLDA cards used in this study contained primers, hydrolysis probes and PCR components for a selected range of gene targets on a micro-fluidic card set-up (see Chapter 2.4.4). This allowed us to perform expression analysis of a moderately large number of genes by RT-qPCR. Here, I will summarise the function and explain the selection of some of the genes on the two card set-ups used, though note that the cards were used for other projects so may include genes not directly relevant to this study. Please see Table 2.5 and Table 2.6 in Materials and Methods for a full list of all genes probed by the cards.

The first card focussed on genes relevant to inflammation and airway remodelling and used *RN18S1* (18S rRNA), *ACTB*, *GAPDH*, *HPRT1* and *PGK1* as candidate reference genes.

Interleukins (IL) form a large and varied group of immunomodulatory cytokines and some are of particular interest for asthma and inflammatory respiratory disease research; several were included on the card: IL-1α and -1β are pro-inflammatory...
cytokines, primarily produced by macrophages and epithelial cells, which contribute to co-ordination of innate and acute phase immune responses. IL-4, IL-5, IL-6 and IL-13 are T\(\text{H}_2\) cytokines so are notable in asthma research. IL-4 and IL-13 are closely related interleukins which are important in driving T\(\text{H}_2\) cell development and IgE class switching in B-cells. IL-5 stimulates development and activation of eosinophils so is of relevance to asthma and atopic disease, while IL-6 is known to mediate fever and acute phase response, but can also act to reduce tissue inflammation and is thought to be important in airway remodelling. Expression of genes encoding all these interleukins was assessed. A related, but less well studied cytokine, oncostatin M (OSM) was also included. CXCL8 (IL-8) is a chemokine which recruits neutrophils and promotes angiogenesis at the site of inflammation. IL-9 activates mast cells and stimulates T\(\text{H}_2\) development so has clear significance for allergic disease. IL-10 is a regulatory cytokine which is secreted by T\(\text{H}_2\) and T\(\text{reg}\) cells and may have disrupted function in airways disease, while IL-18 induces IFN-\(\gamma\) production and promotes a T\(\text{H}_1\) response (Murphy, 2012). Genes for the pro-inflammatory cytokine, TNF\(\alpha\) and the related Fas ligand, as well as the cytokine regulator, suppressor of cytokine signalling 1 (SOCS1), were also included. Expression of genes for several other known inflammatory markers and mediators was studied, including NF\(\kappa\)B-1, -2 and -3, interferon-\(\gamma\) and nitric oxide synthase 2 (NOS2).

Several chemokines from the CCL, CXCL and CX\(3\)CL families were included in the card since they are key recruiters of immune cells to sites of injury and inflammation, events which are dysregulated in asthma. They exert their effects via GPCRs and can recruit a range of circulating leukocytes; those studied have targets including eosinophils, neutrophils, basophils, T-cells, NK cells and macrophages (Murphy, 2012). GM-CSF is a structurally unrelated chemokine which promotes development of granulocytes and macrophages and is up-regulated in asthmatics (Bafadhel et al., 2012). MIF, a regulator of macrophage migration, was also studied.

In asthma, structural changes to the airway including alteration of the extracellular matrix (ECM) can occur. Several components and modulators of ECM architecture were among targets on the card, including several collagens, fibronectin, vimentin, cadherins, chitinase, matrix metalloproteinases and their inhibitors. In addition to ECM components and modulators, several targets involved in cell-matrix interactions were
assessed, namely ICAM1, ADAM19, LAMP2, THBS1 and SPP1, since they have potential implications for lymphocyte recruitment or airway remodelling. Genes for growth factors and their modulators were also studied as they have functions relevant to remodelling and some have immunoregulatory functions; TGFB1, CTGF, EGF, HGF and its inhibitor SPINT1, FGF, BMP4, BMPR2, GREM1, WISP1, VEGF and PDGF.

Three PDE4 family members were assessed. These cAMP-specific phosphodiesterases are important regulators of smooth muscle tone and inflammation (Houslay and Adams, 2003). The cAMP system is the target of the main treatment used to combat the hypercontractile phenotype observed in asthmatic patients.

Several other gene targets whose products have a range of different functions, including cell signalling molecules, transcription factors, enzymes and glycoproteins, were also included in the study since evidence existed in the literature to suggest a possible link with asthma or other inflammatory airways disease. These include EDN1 (Pegorier et al., 2007), HMOX1 (Raval and Lee, 2010), MUC5AC (Kirkham et al., 2002), NFE2L2 (Fitzpatrick et al., 2011), NR1P1 and -2, SEMA6C, SEMA7A (Smith et al., 2011), PIM1 (Shin et al., 2012), PLAUR, SERPINC2, SERPINE1 (Swaisgood et al., 2007), SNAI1 and SNAI2 (Volckaert et al., 2011).

The second card focused on genes relevant to LPA signalling and metabolism and had eight candidate controls; RN18S1, ACTB, GAPDH, HMBS, HPRT1, PGK1, PPIA and TFRC. LPA is known to be produced by two distinct routes - by removal of the choline group from lysophosphatidylcholine (LPC) by the lysophospholipase D, autotaxin, or by removal of the head group from a phospholipid by a phospholipase D to form phosphatidic acid (PA), which is then converted to LPA by removal of a fatty acid group by a phospholipase A1/2 (PLA1/2; Aoki et al., 2008). In addition to autotaxin (ENPP2), other enzymes with a demonstrated physiological role in LPA production were included in the study. Lecithin cholesterol acyl transferase (LCAT) converts phosphatidylcholine (PC) in serum to LPC, thus providing a substrate for autotaxin (Aoki et al., 2002). A number of PLA1/2 enzymes have been reported to play a role in LPA generation by breaking down PA, potentially from the plasma membrane, into LPA. LIPH and LIPI encode the membrane-bound PA-selective PLA1s, mPA-PLA1-α and -β respectively, while PLA2G2A and PLA2G4A encode the secretory PLA2 group IIA (sPLA2-IIA) and the cytosolic PLA2 (cPLA2). The phosphatidylserine (PS)
selective PLA₁, PS-PLA₁ (encoded by PLA1A) can contribute to serum LPA production by providing lyso-PS substrate for autotaxin (Aoki et al., 2002). In addition to these targets involved in LPA production, three lipid phosphate phosphatases (LPPs) which breakdown LPA were also probed (PPAP2A-C).

Finally, a number of genes related to signalling pathways known to be activated by LPA were included in the study. In addition to the six confirmed human LPA receptors (LPAR1-6), several components of the cAMP signalling pathway were studied including adenylyl cyclases 1-9 (ADCY1-9), G protein α-subunits involved in regulation of adenylyl cyclase activity (GNAS and GNAI1-3) and the phosphodiesterases 4A and 4B.
3.2. Results

3.2.1. Identification of targets of interest for quantitative expression analysis using TLDA technology

3.2.1.1. Control experiments to ensure the absence of genomic DNA contamination in cDNA samples and consistency of sample isolation.

Genomic DNA contamination was assessed by performing RT-qPCR experiments on all RT+ and RT- cDNA samples using primers for 18S rRNA which would allow amplification of the rat or human sequence. Since 18S rRNA is abundant in all mammalian cell types, this would provide a sensitive test for contamination. cDNA samples were generated from RNA isolated from clinically characterised cultured hASM cells from control or asthmatic donors. While a DNAse I treatment was performed as part of the RNA isolation, it was necessary to check for any genomic DNA (gDNA) contamination which could cause false positive results in the study. When cDNA samples were prepared from the isolated RNAs by reverse transcription, RT- control samples, that were prepared without reverse transcriptase enzyme, were also prepared for each sample. If there is no gDNA contamination in the prepared RNA, the RT- control samples will contain no DNA. Of the cDNAs used, all RT+ samples tested positive for 18S rRNA, with a C_t of 12.18 ± 0.14 (mean ± s.e.m.), while most RT- samples didn't cross the threshold at all and those that did had a much higher C_t of 33.84 ± 0.12 (mean ± s.e.m.). Figure 3.1 shows a representative amplification plot and the mean data.

In order to test the consistency of cDNA sample composition, RNA was harvested from two preparations of cultured hASM cells from the same asthmatic donor in parallel. The cDNAs prepared from these samples were assessed for expression of approximately 40 gene targets using an LPA TLDA. A comparison of ΔC_t values generated reveals a strong correlation between the two preparations, with a Pearson's r value of 0.989, across a broad range of values (Figure 3.2).

3.2.1.2. Validation and selection of reference genes for TLDA

Good reference genes are stably expressed across all samples, despite experimental variables, in order to be used for accurate analysis and interpretation of RT-qPCR data.
Figure 3.1

A. A representative amplification plot from experiments to test for the presence of contaminating genomic DNA in cDNA samples by RT-qPCR amplification of 18S rRNA. The dotted line represents the signal threshold. B, a table summarising the mean data. Ct values for RT+ and RT- samples are significantly different as assessed by t-test, p < 0.0001.

<table>
<thead>
<tr>
<th>Proportion of samples with 18S amplification</th>
<th><strong>C_t</strong></th>
<th>Mean</th>
<th>± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT+</td>
<td>26 / 26</td>
<td>12.18</td>
<td>± 0.14</td>
</tr>
<tr>
<td>RT-</td>
<td>3 / 26</td>
<td>33.84</td>
<td>± 0.12</td>
</tr>
</tbody>
</table>
Figure 3.2

A scatterplot illustrating the correlation between Δ Ct values for two cDNA samples prepared from two different cultures derived from the same asthmatic donor. The samples were prepared in parallel and run on an LPA TLDA. Each data point represents one gene target. The Pearson’s r value for the correlation is inset.
In this work, multiple reference genes were used to analyse the data by the relative quantification method as recommended by Vandesompele et al., (2002) in order to reduce the weighting given to any one reference gene and to increase accuracy. Figure 3.3 and Figure 3.4 show amplification plots for the candidate reference genes that were included in the TLDAs used. There is good stability of C\textsubscript{t} values across most samples for all the candidate reference genes (Figure 3.5 and Table 3.1), but with three clear anomalies; two very high C\textsubscript{t} values for RN18S1 on the inflammation TLDA (samples A109 and A169) and one slightly low C\textsubscript{t} for GAPDH on the LPA TLDA (sample A109). The outlying results can be easily identified on the amplification plots (Figure 3.3A and Figure 3.4C) as they have a different reaction profile than other surrounding samples.

3.2.1.3. **TLDA card experiments to identify potential targets of interest.**

cDNA samples were assayed using inflammation and LPA TLDA cards which probe for expression of a wide range of gene targets to enable identification of potential genes of interest for follow-up in the more intensive and quantitative RT-qPCR study that would follow. Figure 3.6 and Figure 3.7 show volcano plots summarising the resulting TLDA data; these plot the difference in expression between the control and test group against the significance of that comparison, allowing easy identification of targets of interest. Since high C\textsubscript{t} values cannot be reliably interpreted, the data were analysed in two ways; either including or excluding values above 32 cycles. Where the high values were included and a sample didn't cross the threshold, the C\textsubscript{t} for that sample was set at 40, the maximum number of cycles performed. Samples were grouped in a number of ways according to disease severity; all control samples were compared to all asthmatics, severe and moderate asthmatics or severe asthmatics only. Any genes that crossed the significance barrier of p = 0.05 (= -log\textsubscript{10} (1.3)) were assayed in the follow-up study (labelled data-points in Figure 3.6 and Figure 3.7). Note that the majority of selected genes were identified in several analyses of the data, though the pattern was different for each gene. For example, expression of SOCS1 is shown to be significantly decreased in asthmatics compared with controls, regardless of whether C\textsubscript{t} values above 32 cycles are included or not and regardless of how the groups are defined. On the other hand, LIPH is significantly increased in asthmatics, regardless of whether maximum
Figure 3.3
Amplification plots for the candidate reference genes for inflammation TLDAs. ∆Rn represents fluorescence signal over baseline and is presented on a logarithmic scale against the number of PCR cycles. The red line indicates the signal threshold, which is set at 0.2 for all reactions. Amplification traces from each of the 20 cDNA samples used are overlaid for each gene target. A–E show amplification plots for RN18S1, ACTB, GAPDH, HPRT1 and PGK1, respectively.
Figure 3.4

Amplification plots for the candidate reference genes for LPA TLDAs. Δ Rn represents fluorescence signal over baseline and is presented on a logarithmic scale against the number of PCR cycles. The red line indicates the signal threshold which is set at 0.2 for all reactions. Amplification traces from each of the 26 cDNA samples used are overlaid for each gene target. A–H show amplification plots for RN18s1, ACTB, GAPDH, HMBS, HPRT1, PGK1, PPIA and TFRC, respectively.
Figure 3.5

Graphs showing C_t values for candidate reference genes for each cDNA sample to allow easy identification of anomalous values and selection of reference genes to use in the analysis. A shows results for the inflammation TLDA, while B shows results for the LPA TLDA.
### Table 3.1

Table showing average $C_t$ values and gene stability scores for the candidate reference genes for A, inflammation and B, LPA TLDAs. Values quoted are mean ± s.e.m. 'n' values are 20 and 26 for A and B, respectively. Gene stability scores are calculated as described in Vandesompele et al. (2002).
A. All asthmatics, maximum values included

B. All asthmatics, maximum values excluded

C. Severe and moderate asthmatics, maximum values included

D. Severe and moderate asthmatics, maximum values excluded
Figure 3.6
Volcano plots illustrating the variation in \( C_t \) values from inflammation TLDAs with donors grouped in various ways. Each data-point represents a gene target; its position is determined by the difference in the means of the groups (x-axis) and significance of this comparison (y-axis). Threshold values were set at a fold change of \( \pm \log_2(1) \) or significance of \( p = \log_{10}(-1.3) = 0.05 \), as indicated by lines on plots. A and B, controls compared against all asthmatics. C and D, controls compared against severe and moderate asthmatics. E and F, controls compared against severe asthmatics. A, C and E, all \( C_t \) values were included for analysis. B, D and F, \( C_t \) was capped at 32 cycles and higher data points excluded from analysis.
A. All asthmatics, maximum values included

B. All asthmatics, maximum values excluded

C. Severe and moderate asthmatics, maximum values included

D. Severe and moderate asthmatics, maximum values excluded
Figure 3.7
Volcano plots illustrating the variation in C\textsubscript{t} values from LPA TLDAs with donors grouped in various ways. Each data-point represents a gene target; its position is determined by the difference in the means of the groups (x-axis) and significance of this comparison (y-axis). Threshold values were set at a fold change of ±\log\textsubscript{2}(1) or significance of \( p = \log\textsubscript{10}(-1.3) = 0.05 \), as indicated by lines on plots. A and B, controls compared against all asthmatics. C and D, controls compared against severe and moderate asthmatics. E and F, controls compared against severe asthmatics. A, C and E, all C\textsubscript{t} values were included for analysis. B, D and F, C\textsubscript{t} was capped at 32 cycles and higher data points excluded from analysis.
values are included or not, but only where the asthmatic group is limited to donors exhibiting severe disease.

3.2.2. Quantitative expression analysis of the identified genes of interest using RT-qPCR and standard curve analysis

Expression levels of a number of genes of interest identified from the TLDA experiments described above were investigated using samples derived from low-passage hASM cells from control and asthmatic donors by RT-qPCR and the standard curve method of analysis. Standard curves, prepared using a range of starting amounts of a calibrator cDNA sample, were generated for each primer and hydrolysis probe set (Figure 3.8). The slopes of all the lines are close to the ideal of -3.3 which indicates 100 % efficiency of the PCR (since each PCR cycle would then result in a doubling of amplicon), making them suitable for quantitative analysis. Note that the lines occur at different levels on the y-axis, illustrating a range of abundances of the gene targets in the calibrator cDNA sample. For example, the line for GAPDH has a y-intercept of 22.2 cycles (Figure 3.8A), indicating relatively high abundance of GAPDH transcript in the calibrator sample, whereas LIPH (Figure 3.8F) has a y-intercept of 34.7 cycles, indicating lower amounts of LIPH transcript. All but three of the standard curves (those for ADCY8, RAPGEF3 and GNAS; Figure 3.8K-M) have correlation coefficients ($r^2$ values) of $> 0.98$. Such a strong correlation is both expected and required for quantitative analysis. The lines for the three targets with less strong correlations also have the highest y-intercepts at 38.6, 36.6 and 38.5 cycles for ADCY8, RAPGEF3 and GNAS respectively, indicating low abundances in the calibrator sample.

The abundance of each gene transcript was calculated from the appropriate standard curve for 11 control and 10 asthmatic donor cDNA samples to enable comparison of gene expression levels between donors. Asthmatic and control donors were grouped and compared so that disease dependent changes in expression of the genes of interest could be identified (Figure 3.9). Statistical testing comparing all donors revealed no significant differences in expression of any of the genes assessed between asthmatic and control donors. Note that for IL1A, MIF and PDE4B, exclusion of the outlying samples does not yield significant results. Limiting the asthmatic samples to just those with a severe or moderate phenotype (6 donors) reveals a significant difference between the
A. GAPDH

\[ y = -3.37x + 22.2 \]
\[ r^2 = 0.996 \]

B. SOCS1

\[ y = -3.32x + 30.5 \]
\[ r^2 = 0.994 \]

C. IL1A

\[ y = -3.46x + 33.5 \]
\[ r^2 = 0.987 \]

D. MIF

\[ y = -3.35x + 27.6 \]
\[ r^2 = 0.993 \]

E. CCL11

\[ y = -3.45x + 34.7 \]
\[ r^2 = 0.982 \]

F. LIPH

\[ y = -3.50x + 29.7 \]
\[ r^2 = 0.997 \]

G. PPAP2C

\[ y = -3.41x + 32.0 \]
\[ r^2 = 0.994 \]

H. PDE4B

\[ y = -3.50x + 29.7 \]
\[ r^2 = 0.997 \]
Figure 3.8
Graphs showing standard curves generated for each primer and probe set used in RT-qPCR experiments. Standard curve reactions were prepared in triplicate and the graphs plot mean C<sub>t</sub> value (± s.e.m.) against total amount of input DNA in ng on a logarithmic scale. Line equations and R<sup>2</sup> values are inset. Line equations take the format ‘y = mx + c’, where ‘m’ is the slope of the line and ‘c’ is the y-intercept. A-M show the standard curves generated for primer probe sets for GAPDH, SOCS1, IL1A, MIF, CCL11, LIPH, PPAP2C, PDE4B, PDE4D, SPHK2, ADCY8, RAPGEF3 and GNAS respectively.
A. SOCS1

B. IL1A

C. MIF

D. CCL11

E. LIPH

F. PPAP2C

G. PDE4B

H. PDE4D

I. SPHK2
**Figure 3.9**

Scatterplots showing quantity of gene target relative to *GAPDH* from the final analysed results of RT-qPCR experiments. Each data point shows the mean of one cDNA sample, which was assayed in triplicate and represents one donor. Lines and error bars show mean ± s.e.m. for the groups. Comparison of the groups by *t*-test showed that relative amounts were not significantly different between the groups for any gene target. A-I show the results for *SOCS1, IL1A, MIF, CCL11, LIPH, PPAP2C, PDE4B, PDE4D* and *SPHK2*, respectively.
groups for expression of $PDE4B$ ($p = 0.030$) with relative abundances of $0.977 \pm 0.126$ and $0.526 \pm 0.103$ for controls and asthmatics, respectively (Figure 3.10A). Limiting the asthmatic donors further to only those with a severe phenotype (4 donors) produced data showing a significant reduction in expression of $PPAP2C$ in asthmatics compared to controls, from $1.823 \pm 0.376$ to $0.402 \pm 0.148$ ($p = 0.046$; Figure 3.10B). However, the significance of the comparison between control and asthmatic donors' expression of $PDE4B$ is lost when the asthmatic group is reduced to the severe phenotype only ($p = 0.078$; Figure 3.10C).
Figure 3.10

Scatterplots showing quantity of gene target relative to GAPDH from the analysed results of RT-qPCR experiments where significant results were observed. Each data-point shows the mean of one cDNA sample, which was assayed in triplicate and represents one donor. Lines and error bars show mean ± s.e.m. for the groups. In A, the asthmatic group is limited to donors with moderate or severe phenotypes, while in B and C, the group is further narrowed to severe only. * indicates p ≤ 0.05.
3.3. **Discussion**

The aim of this Chapter was to identify genes with disease-dependent changes in expression maintained in a cultured hASM cell system. This was done by a two-stage approach that used two different formats of RT-qPCR using hydrolysis probes for detection. Firstly, a broad expression screen was performed to identify potential targets of interest using TLDAs and secondly, the identified targets were more quantitatively followed up using individual primer and hydrolysis probe sets and the standard curve method of analysis for RT-qPCR.

3.3.1. **Identification of genes of interest by RT-qPCR with a TLDA format**

3.3.1.1. **Assessment of sample quality**

In the initial stage, expression of over 40 genes was assayed semi-quantitatively in a high-throughput manner using a TLDA system. Two cards were used; one which assayed for gene targets relevant to inflammation and remodelling and another which assayed for targets relating to LPA metabolism and signalling (See Table 2.5 and Table 2.6 for the full list of gene targets assayed). With careful application, this RT-qPCR method can be very powerful, but meaningful interpretation of the data relies on two key criteria; quality of the cDNA samples and quality of the endogenous controls used for analysis (Thellin *et al.*, 2009).

It was important to ensure that cDNA sample quality was adequate for this work and that the cDNA samples obtained accurately and reproducibly represented the expression profile of the cultured hASM cells they were derived from. Quality of cDNA is mostly affected during the RNA isolation and reverse transcription stages. RNA can quickly become degraded by RNases during isolation. Total RNA was prepared from cultured cells in this study, rather than a complex tissue, so it was not anticipated that excessive degradation would be a problem since cell lysis and RNA extraction could be done very quickly and efficiently. Additionally, reverse transcriptions were performed soon after RNA isolation to minimise RNA storage. The reverse transcription stage can be performed using two different priming methods; either using oligo-d(T)s, which prime mRNAs from their poly-A tail, or alternatively, using random hexamers which prime from random points along the RNA sequence. The exclusive use of oligo-d(T)s enables
restriction of the cDNA library to copied mRNAs. This would preclude the use of rRNAs as reference genes and can produce truncated cDNAs where the reverse transcriptase hasn't completed transcription. Random hexamers ensure complete transcription of the full gene length and also of all cellular RNAs. Here, a mixture of oligo-d(T)s and random hexamers was used to ensure complete transcription of RNA, so that the cDNA fully represents all the RNA present in the cell sample. For this study, cDNAs needed to represent the RNA in the cell sample and be of good enough quality to allow amplification of short amplicons for detection. This was clearly demonstrated by the reliable and uniform amplification of sequences for several candidate reference genes for all the samples tested, across a range of genes with a range of expression levels (Figure 3.3 and Figure 3.4). Reproducibility of sample preparation was illustrated by the strong correlation of expression levels (ΔCt values) of a range of genes determined by assay of two samples prepared from two cultures of hASM cells from one donor (Figure 3.2). Critically, analysis of RT-control samples showed that cDNA samples were free from gDNA contamination (Figure 3.1), though many of the primer and probe sets span intron boundaries to provide an additional safeguard against detection of gDNA.

3.3.1.2. Selection of reference genes

For meaningful interpretation of RT-qPCR data by the relative quantification method used here, the reference genes used for normalisation of the data must be uniformly expressed across all the samples in a manner that is not subject to variation in the experimental conditions studied, in this case, disease phenotype. Strategies for selection and validation of endogenous controls for RT-qPCR have been extensively studied and improved over the last decade (Thellin et al., 2009). It is easy to assume that "housekeeping genes" are expressed homogeneously across all cell types, regardless of experimental conditions, but in fact, ideal controls vary depending on the tissue or cell type studied and the experimental conditions (Vandesompele et al., 2002). Several studies have brought the reliability of historically used internal controls into question (Dheda et al., 2004). For example, tissue-dependent variability in expression of GAPDH and HPRT1 has been demonstrated, while ACTB expression was found to vary among a group of leukaemia patient tumour samples (Huggett et al., 2005). In this work, the method for normalisation of RT-qPCR data proposed by Vandesompele et al.
(2002) was used. This method provides a measure for gene stability based on comparisons of the variations between pairs of candidate controls, to enable selection of endogenous controls. With the exception of two anomalous results for RN18S1 from the inflammation card and one for GAPDH from the LPA card which, on inspection of the amplification plots, appear to be due to failed reactions rather than variation in starting material, expression of all candidate reference genes was reassuringly consistent. RN18S1 and GAPDH were excluded from the inflammation and LPA cards respectively and the remaining four and seven reference genes, which showed good uniformity of expression, were used to generate the normalisation factor used to calculate the relative expression values for the targets studied.

3.3.1.3. Analysis and interpretation of TLDA data

The quantity of TLDA data produced presented some difficulties for analysis and interpretation. Volcano plots were used to enable easy identification of genes that had large (≥ 2-fold) and/or significant (p ≤ 0.05) differences in expression between the control and asthmatic samples tested. An additional compounding factor was that the data could be legitimately analysed in a number of different ways. To avoid changes which depended on a severe disease phenotype being masked by the expression profile of more mild disease sufferers, which could potentially tend more towards that of control donors, the asthmatic donors were grouped in three different ways for analysis: all, moderate and severe, severe only. It is important to note that it was the same data analysed using the different donor groupings so that 'n' values were decreased with each move towards a severe-only phenotype. This presented the potential problem of increasing the magnitude of change while also increasing the magnitude of error for comparisons. This is particularly relevant for this study since variability between 'n' values was expected to be reasonably high as this represents variability in expression profile between different human beings. In addition to using different donor groupings to analyse the data, two different approaches were taken for defining the lower limit of detection for the assay. The sensitivity of PCR is one of its key strengths as a method, but this increases the possibility of false positive results caused by minute levels of contamination. As such, Ct values above ~35 cycles are generally considered to be unreliable and represent very little or no transcript in the initial sample. Bearing this in mind, data-points above 32 cycles were omitted in one set of analyses. This, however,
increased the likelihood of false negative results since it would preclude any comparison between a group with some level of expression and another with very little. Therefore, the data were also analysed including all data points and assigning the maximum $C_t$ value (40) to any which were below the level of detection. The combination of these approaches resulted in the data from each card being analysed 6 ways and provided an inclusive approach to selection of genes for follow-up. Any gene whose comparison of expression between groups broke the significance barrier for any of the analyses was selected for follow-up, though it is worth noting that all targets selected achieved this in more than one analysis.

3.3.2. Quantitative expression analysis of selected genes by RT-qPCR using hydrolysis probes

For the second stage of this study, the standard curve method of quantification was used. This is more quantitative than the method used to analyse the larger number of genes in the TLDA work since it takes into consideration the amplification efficiency of each assay employed and incorporated more experimental replicates than was possible in the higher throughput method.

Good standard curves were not achieved for three of the genes selected for follow-up - ADCY8, RAFGEF3 and GNAS. Notably, these were also the targets with the highest standard curve $y$-intercepts, at 38.6, 36.6 and 38.5 cycles respectively, which indicate that low levels of these transcripts were present in the calibrator sample, preventing accurate quantification. Since the calibrator sample was composed of cDNA generated from the same cell type, of the same origin as the cDNA samples used in the study, it was anticipated that expression below the level of detection in the calibrator widely indicates that expression would be equally low in the samples. Therefore, study of these targets was ceased at this point.

For the remaining nine targets studied, expression was not significantly altered with disease phenotype where all donors were compared. Restriction of the asthmatic group to more severe phenotypes revealed a significant down-regulation of PDE4B in severe and moderate asthmatics compared to controls. The significance of this comparison was lost when the asthmatic group was reduced to only severe phenotypes, though inspection of the scatterplots might suggest that this is due to the increase in error
associated with the reduction in 'n' number. A further, larger study would be needed to verify these results. Phosphodiesterases have long been studied in relation to asthma, owing to their ability to regulate cellular cAMP levels and consequently, smooth muscle tone and immunomodulators affected by cAMP (Mehats et al., 2003). Since asthma therapies promote cAMP production by stimulating β2-adrenoceptors, it was surprising to observe a down-regulation of PDE4B in this group of severe and moderate asthmatics. It is possible that the observed down-regulation represents a compensatory mechanism to alleviate hypercontractility and inflammation in these cells, though this is really speculation. Few studies have investigated PDE expression in hASM cells from asthmatic donors; one study investigated expression of the PDE4D isoform by RT-qPCR and observed no basal difference between asthmatic and control groups (Niimi et al., 2012). I have also observed no change in PDE4D expression in the two groups, though no other groups have investigated expression of the PDE4B isoform in asthmatic ASM. Investigations of PDE4B expression have focused on the CNS, since it has been associated with psychiatric disease (Millar et al., 2005), though some studies have found a role for PDE4B function in airways inflammation. PDE4B can regulate TLR signalling in mouse macrophages (Jin et al., 2005). Peripheral blood leukocytes from PDE4B−/− mice (Jin and Conti, 2002), or isolated from humans and treated with PDE4A/B inhibitors (Manning et al., 1999), show reduced TNFα production in response to LPS. Work in the respiratory field has focussed on the PDE4D isoform for a number of reasons. Studies in PDE4D+/− mice have revealed that this isoform accounts for most of the PDE activity in isolated mouse trachea and almost all the PDE activity which is sensitive to the PDE4 selective inhibitor, rolipram (Mehats et al., 2003). PDE4D knockout significantly inhibits tracheal contraction in response to the cholinergic agonist, carbachol, to the same extent as rolipram treatment of trachea from wild-type mice (Mehats et al., 2003) and ablates methacholine-induced airway hyperresponsiveness in mice (Hansen et al., 2000). However, despite seeming to account for most, if not all, PDE regulated contractility in trachea, PDE4D knockout had no effect on allergen-induced airway inflammation (Hansen et al., 2000), despite the well established anti-inflammatory effects of PDE4 inhibitors (Giembycz and Newton, 2011). It is possible, therefore, that the relatively low levels of PDE4B, found in ASM could have distinct roles from PDE4D, perhaps in a distinct signalling compartment or pool. The roles of individual PDE isoforms in ASM cells are not currently well
understood, so it is impossible to say what the functional and physiological consequences of the observed downregulation of PDE4B in severe and moderate asthmatics might be without further study.

Expression of PPAP2C, which encodes lipid phosphate phosphatase 2 (LPP2, formerly known as phosphatidic acid phosphatase type 2C, PAP2c) was significantly down-regulated in severe asthmatic donors compared with controls. In contrast to PDEs, literature regarding LPP2 is sparse, all the more so in the context of respiratory disease, so interpretation of the significance of this finding is difficult. It was included in this study for the potential impact its expression could have on LPA levels. Indeed, in vitro studies have shown that all 3 members of the LPP family are capable of breaking down LPA as well as the other bioactive lipids PA, S1P and C1P (Roberts et al., 1998). The balance between autotaxin and LPP activities is thought to control circulating LPA concentrations (Samadi et al., 2011) and it is feasible that down-regulation of an LPP could increase the strength and duration of LPA signalling. In vivo studies have shown that LPP1 can regulate circulating LPA levels, as hypomorph mice with reduced Ppap2A expression increase the half-life of circulating LPA to 12 min from 3 min in control animals (Tomsig et al., 2009). Furthermore, studies on LPP1 indicate that LPP activity can modulate LPA signalling since LPP1 over-expression can attenuate LPA-induced activation of ERK, PLD, Ca^{2+} mobilisation and proliferation of fibroblasts (Jasinska et al., 1999). However, LPP2 is the least well studied of this family of phosphatases, perhaps because it has the most restricted expression pattern of them (Hooks et al., 1998), and only a handful of publications address the physiological functions of this particular subtype. One such study exogenously over-expressed LPP2 in rat fibroblasts and identified a subsequent mistiming of progression into S-phase of the cell cycle, which was found to be dependent on the phosphatase catalytic activity of LPP2 (Morris et al., 2006). This study was not able to correlate the effect with measurable changes in LPA concentration, though such measurements present substantial technical difficulty. A mouse PPAP2C knockout appears phenotypically normal and fertile, yielding little further insight into LPP2 function (Zhang et al., 2000). It is plausible that the observed downregulation of LPP2 in the severe asthmatics studied could result in exaggerated LPA signalling at these cells, though much more work would be needed to fully understand these results.
3.3.3. **Concluding remarks and future directions**

In some respects, the most remarkable result of this study is that there are any transcriptional changes maintained in the cultured conditions. These are unstimulated cells removed from their inflammatory milieu so perhaps these represent some of the more hardwired changes that occur in asthma. This is highlighted by the fact that changes are only observed in the more severe phenotypes. It would be interesting to investigate the expression profile of hASM cells upon stimulation with cytokines which are more abundant in the asthmatic lung, or with sputum samples or broncho-alveolar lavage fluid from asthmatic donors. For the purposes of this study, it would be particularly interesting to investigate the effects of stimulation with LPA. The method employed here, whereby a medium scale screen is assayed before reduction to a few genes of interest has promising potential for investigations of this sort and allows efficient use of valuable biological material.
CHAPTER 4. Pharmacological profiling of LPA receptor subtypes
4.1. Introduction

The aims of this Chapter are to characterise the actions of a range of LPA species and other lipids with reported agonist or antagonist activity at a range of LPA receptors. Please see Table 2.1 for structures of LPA species used and Table 2.2 for structures of lipid agonists and antagonists used.

While it is thought that the LPA receptors bind LPA at the phosphate moiety (Jalink et al., 1995; Santos et al., 2000), it is interesting to note that some studies have suggested that different LPA receptors might have differing preference for the various LPA species. Bandoh et al. (2000) measured LPA-induced changes in intracellular Ca²⁺ concentration in baculovirus-infected Sf9 insect cells expressing each of the Edg family LPA receptors to assess differential activation of these three LPA receptors by different LPA species. They tested saturated and unsaturated LPAs with different acyl chain lengths joined to the glycerol backbone at either the sn-1 or sn-2 position and found that all species tested were approximately equipotent at the LPA₁ and LPA₂ receptors with the exception of 12:0, which was not active at any of the receptors, while LPA₃ receptors showed a marked preference for unsaturated LPAs with the sn-2-acyl species being consistently more active than their sn-1-acyl counterparts. This work has been challenged by findings that suggest the receptors have the same rank-order of LPA potencies as assessed by [³⁵S]GTPγS binding in transfected RH7777 membranes (Im et al., 2000); but is supported by findings that unsaturated, but not saturated, LPAs are able to induce neointimal hyperplasia, vascular remodelling and vascular smooth muscle cell dedifferentiation, as measured by changes in cell morphology and suppressed expression of the smooth muscle cell marker genes caldesmon and calponin (Hayashi et al., 2001; Yoshida et al., 2003). It has also been suggested that various biological fluids can differentially activate the LPA receptors, though it was the associated protein carriers that were suggested to be implicated in this (Hama et al., 2002). While sn-1-acyl forms are more common and chemically stable, sn-2 forms are known to exist physiologically and it has been reported that they are enriched in the fluid from ascites formed in ovarian cancer (Xu et al., 1995). Also, LPA levels are increased after segmental allergen challenge and polyunsaturated forms, including 22:6 and 20:4, are markedly more increased relative to total LPA levels (Georas et al., 2007).
There is a notable paucity of receptor subtype-specific ligands in the lysophospholipid signalling field which has hampered the elucidation of the physiological roles that the various LPA receptor subtypes play. The first reported receptor ligand, N-acyl ethanolamine phosphoric acid (NOEPA) was found to have similar potency to LPA in stimulating platelet aggregation and $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding via the LPA$_1$ receptor (Sugiura et al., 1994). A related compound, N-acyl serine phosphoric acid (NASPA) has been reported to antagonise LPA-induced membrane currents in electrophysiological recordings of Xenopus oocytes (Bittman et al., 1996; Liliom et al., 1996), but acted as an agonist in a study using the human breast cancer cell-line MDS MB231 (Hooks et al., 1998). This discrepancy might be attributable to differences between the amphibian and mammalian LPA receptors, or because the studies all used endogenous LPA receptors of unknown subtype.

Using NOEPA as a ‘parent compound’ Lynch and MacDonald developed several lipid-based LPA receptor agonists and antagonists. VPC 31143 has been shown to act as an agonist with greater potency than LPA (18:1) at LPA$_1$ as assessed by $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding, while its S-enantiomer VPC 31144 had several-fold lower potency (Heise et al., 2001). The LPA analogue, (2S)-3-[((hydroxymercaptophosphinyl)oxy]-2-methoxypropyl ester, 9Z-octadecenoic acid (2S)-OMPT), has been reported to be an LPA$_3$-selective agonist, being several log orders more potent in eliciting Ca$^{2+}$ responses or promoting $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in Sf9 and HEK293 cells transiently transfected with LPA$_3$, than LPA$_1$ or LPA$_2$ (Hasegawa et al., 2003). VPC 12249 has been reported to inhibit LPA-induced $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in HEK293 cells transfected with LPA$_1$ or LPA$_3$, but not LPA$_2$ and so has been designated an LPA$_{1/3}$-selective antagonist (Heise et al., 2001). These compounds have been found to affect functional responses: OMPT increased ischaemic reperfusion injury in mice as measured by plasma creatinine concentrations, while pre-administration of VPC 12249 reduced this effect (Okusa et al., 2003). Fatty acid phosphates, such as dodecyl fatty acid phosphate (DDP), which are structurally similar to LPA, but lack a glycerol or similar link between the phosphate and fatty acid moieties, have been found to act as competitive antagonists at LPA$_1$ and LPA$_3$, but as agonists at LPA$_2$ in Ca$^{2+}$ assays in RH7777 cells transfected to express the various LPA receptors (Virag et al., 2003). VPC 32179 and its S-enantiomer VPC 32183 were found to be more potent antagonists than the parent compound VPC 12249 and act at LPA$_1$ and LPA$_3$, but have no activity at LPA$_2$ (Heasley et al.,
The latter compounds have been used in functional assays to antagonise LPA-induced responses in primary cells, including human cord-blood mast cell proliferation (Bagga et al., 2004) and interleukin secretion and migration of human fibroblast-like synoviocytes from patients with rheumatoid arthritis (Zhao et al., 2008).

While the generation of these compounds represents a significant advance for pharmacological investigation of the LPA receptors, they have not been well-characterised and are not extensively used within the field. For example, some of the initial characterisation was performed using LPA receptors exogenously over-expressed in cell-types which are known to express endogenous LPA receptors, without appropriate wild-type control experiments being reported (Heise et al., 2001; Heasley et al., 2004). Studies have been performed using a variety of assays, including a $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding assay (Heasley et al., 2004) and $\text{Ca}^{2+}$ mobilisation assays (Virag et al., 2003) in a range of cell backgrounds, including transfected HEK293 (Heise et al., 2001) and RH7777 (Virag et al., 2003) cells. Some studies have reported the effects of ligands on endogenous LPA responses and so cannot be attributed to any particular receptor subtype (Lynch et al., 1997; Hooks et al., 1998). Because of these difficulties I initially wished to screen commercially-available LPA receptor ligands with reported agonist or antagonist activity in cell-lines exogenously expressing various LPA receptors in an LPA receptor-null background.

The expression of LPA receptors appears to be almost ubiquitous. Northern blot analysis of adult human tissue has revealed that LPA$_1$ is predominantly expressed in brain, but can be found in many other tissues, including heart, pancreas, kidney, muscle, the reproductive system, intestine and colon. LPA$_2$ expression is also widespread and particularly high in peripheral blood leukocytes (An et al., 1998). LPA$_3$ also has an extensive expression pattern and can be detected in the reproductive system, heart, lung and pancreas (Bandoh et al., 1999). Similarly, the more recently identified receptor family members have been found to be expressed in several tissue-types (Noguchi et al., 2003; Lee et al., 2006), although LPA$_4$ is highly expressed in the ovary compared to other tissues (Noguchi et al., 2003). LPA receptors have also been detected in a variety of model cell-lines commonly used within the scientific community, including HEK293 and HeLa cells (An et al., 1997; Bandoh et al., 1999). The near-ubiquitous expression of LPA receptors is a significant problem when trying to attribute an LPA-induced
effect to a particular receptor subtype, especially since new receptors in this family may continue to emerge and there are no validated subtype-specific inhibitors. A hematoma cell-line derived from a McArdle rat hepatoma, RH7777, and the rat neuroblastoma cell-line B103 have emerged as the field’s preferred null cell background, since neither is responsive to LPA in a range of cell assays (Fukushima et al., 1998). Although functionally null it should be noted that Northern blot analysis shows that LPA5 is detectable in RH7777 cells (Lee et al., 2006).

Stable RH7777 cell-lines transfected to express human LPA receptors were used in the initial stages of this study. A $[^{35}\text{S}]\text{GTP}_{\gamma} \text{S}$ binding assay was optimised to measure LPA receptor activation in this system. This assay provides a means for quantifying an early functional response of receptor stimulation before amplification or modification further downstream. The canonical mechanism for GPCR activation involves the exchange of GDP for GTP at the $\alpha$ subunit of the G protein $\text{G}_{\alpha\beta\gamma}$ trimer on agonist binding to the receptor and consequent dissociation of activated $\text{G}_{\alpha}\text{-GTP}$ from $\text{G}_{\beta\gamma}$ subunits, which then effect modulations of downstream signalling events. The intrinsic GTPase activity of $\text{G}_{\alpha}$ allows a return to the GDP-bound form and re-association of $\text{G}_{\alpha}\text{-GDP}$ with $\text{G}_{\beta\gamma}$ subunits. $\text{GTP}_{\gamma} \text{S}$ is a hydrolysis-resistant analogue of GTP that is very poorly susceptible to the GTPase activity of the $\text{G}_{\alpha}$ subunit and so an accumulation of $\text{GTP}_{\gamma} \text{S}$-bound $\text{G}_{\alpha}$ subunits in cell membranes can be measured. The use of $[^{35}\text{S}]\text{GTP}_{\gamma} \text{S}$ allows a convenient and readily quantifiable means to measure this accumulation by scintillation counting.

In addition to this, a commercially available rat-derived cell-line, Chem-1, was used in the latter stages of the study. Chem-1 cells over-express the promiscuous G protein, $\text{G}_{\alpha15}$, to permit coupling to the PLC/IP3/Ca2+ pathway and enable easy measurement of receptor activity, as suggested by Offermanns and Simon (1995). Chem-1 cells stably transfected with GPCRs are marketed as "calcium-optimised" drug-screening tools (Millipore) and were used here to screen a range of lipids for their activity at LPA1, LPA2 and LPA3 receptors. SW982 cells were also used as a human LPA receptor model cell line. SW982 cells are human synovial sarcoma-derived fibroblasts which are LPA-responsive.
4.2. Results

4.2.1. Optimisation of a total $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay

A $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay has been optimised in RH7777 cell-lines that over-express various LPA receptors in an otherwise LPA receptor-null background to provide a means for measuring LPA receptor activity. Various assay parameters needed to be optimised since ideal conditions have been shown to vary between systems. This requires optimisation of incubation timings, GDP concentration, membrane protein concentration and $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ concentration.

$[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay time-courses

It was important to ascertain an appropriate incubation time which allows for adequate accumulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ bound $\text{G}\alpha$ in the membrane for detection, without allowing too much basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to occur. Time-course experiments were performed in which RH7777-LPA1 cell membranes were stimulated with different LPA concentrations and total $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ measured at different time-points (Figure 4.1). While the EC$_{50}$ values measured did not differ significantly at any of the time-points (15-180 min) it was found that after longer incubation periods, basal levels of binding increased, decreasing the window in which to observe the consequence of receptor activation. For this reason, incubation times were kept short; to less than 60 min and optimally 30 min. However, when measuring antagonist activity it is necessary to pre-incubate ligands with membranes to allow time for receptor binding. It was found that a pre-incubation time of 30 min and an incubation time of a further 30 min was adequate to measure both LPA-mediated receptor activation and Ki16425-mediated inhibition of the LPA response (Figure 4.2). As has been previously reported in the literature (Ohta et al., 2003; Moughal et al., 2006), Ki16425 was able to reduce $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding below to basal levels (Figure 4.2) possibly indicating inverse agonist activity, or an endogenous LPA stimulatory tone contributing to the observed basal value.

4.2.1.1. Testing for LPA breakdown

LPA is known to be broken down by the lipid phosphate phosphatase LPP1 (Pilquil et al., 2001) and can undergo acyl migration or oxidation of the fatty acyl tail. To ensure that no significant LPA breakdown was occurring during the time-course of the
Figure 4.1

$[^{35}S]$GTP$_\gamma$S binding assay time-courses. A, RH7777-LPA$_1$ membranes were stimulated with various LPA concentrations and incubated with $[^{35}S]$GTP$_\gamma$S binding assay reagents for various times as indicated. B, pEC$_{50}$ values from LPA concentration response curves shown in A. C, Window sizes (maximal response to LPA minus basal value) for LPA concentration-response curves shown in A. One-way ANOVA analysis showed that window sizes at 90, 120 and 180 min were significantly lower than at 30 or 60 min, p ≤ 0.01.
Figure 4.2

LPA concentration-response curve and concentration-dependent inhibitory effects of the LPA receptor antagonist, Ki16425, for $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding at 30 min. The Ki16425 inhibition curve was generated in the presence of 10 nM LPA with a 30 min pre-incubation with antagonist. Observed $pEC_{50}/pIC_{50}$ values are $7.96 \pm 0.06$ and $7.30 \pm 0.02$ for LPA and Ki16425, respectively.
experiment, experiments were performed wherein RH7777-LPA<sub>1</sub> membranes were stimulated with LPA that had either been prepared and then left out at room temperature for 24 hours or freshly prepared (Figure 4.3). To test for biological breakdown the experiments were also performed in the presence or absence of the broad spectrum phosphatase inhibitor, sodium orthovanadate. The responses to LPA left out for 24 hours were indistinguishable from those to freshly prepared LPA (Figure 4.3A), nor did the presence of sodium orthovanadate affect the results (Figure 4.3B). Therefore, LPA is chemically stable within the parameters of the experimental protocol, though biological breakdown is likely to have greater implications in assays that utilise intact cells.

4.2.1.2. Optimisation of assay variables

The [<sup>35</sup>S]GTPγS assay is typically performed in the presence of GDP, which serves to occupy 'empty' Gα subunits and to compete with [<sup>35</sup>S]GTPγS for Gα binding and thus reduce the levels of basal [<sup>35</sup>S]GTPγS binding, allowing optimal measurement of agonist-induced [<sup>35</sup>S]GTPγS binding. The optimum GDP concentration should be determined since this is system-dependent, as is the optimal concentration of [<sup>35</sup>S]GTPγS. The concentration of membrane protein required to achieve optimal [<sup>35</sup>S]GTPγS binding must also be determined, since this depends on receptor expression levels. Assays were performed in the presence of different concentrations of GDP, membrane protein and [<sup>35</sup>S]GTPγS (Figure 4.4). As expected, increasing GDP concentration reduced levels of [<sup>35</sup>S]GTPγS binding, without affecting EC<sub>50</sub> values. While the presence of 10 µM GDP resulted in the lowest basal levels of [<sup>35</sup>S]GTPγS binding, it also produced concentration-response curves with a smaller window of receptor activation. It was therefore concluded that a GDP concentration of 3 µM was optimal, since it provided a compromise of sufficiently low levels of basal binding while retaining an adequate response window. Increasing concentrations of membrane protein or [<sup>35</sup>S]GTPγS increased the magnitude of response, without affecting EC<sub>50</sub> values. A membrane concentration of 20 µg per well and [<sup>35</sup>S]GTPγS concentration of 300 pM were chosen to give a good window of activation. These assay conditions were applied in all further experiments.
Assessment of LPA stability under the employed assay conditions. A, RH7777-LPA₁ membranes were stimulated with LPA that had been freshly prepared, or that had been left at room temperature for 24 h. B, RH7777-LPA₁ membranes were stimulated with freshly prepared LPA in the presence or absence of sodium orthovanadate at a final concentration of 100 µM. C, pEC₅₀ values obtained under the various conditions: no statistically significant differences were observed.
Figure 4.4

Optimisation of assay variables. LPA concentration-response curves generated from [35S]GTPγS binding assays performed using various GDP (A), membrane protein (B) and [35S]GTPγS (C) final concentrations as indicated. EC50 values did not differ significantly under any observed conditions. Responses are presented as percentages of the response observed with 3 μM GDP, 20 μg/well membrane protein or 300 pM [35S]GTPγS.
4.2.1.3. **DMSO and ethanol tolerance**

Since solvents are required to keep some receptor agonists and antagonists in solution, DMSO and ethanol tolerance experiments were performed to ensure this would not affect the assay. RH7777-LPA₁ membranes were stimulated with various LPA concentrations in the presence or absence of DMSO or ethanol. While the presence of ethanol could change the raw CPM values observed, pharmacological parameters, such as the EC₅₀, were not altered significantly by the presence of either solvent (Figure 4.5).

4.2.2. **Effect of pertussis toxin treatment on [⁳⁵S]GTPγS binding in RH7777-LPA₁ membranes**

RH7777-wild-type membranes and RH7777-LPA₁ membranes derived from control or PTx pre-treated RH7777-LPA₁ cells were assayed for an LPA response to determine what, if any, LPA-induced response can be observed for RH7777-wild-type membranes and to determine the contribution of [⁳⁵S]GTPγS-bound Gα to the observed response from RH7777-LPA₁ membranes (Figure 4.6). The mean response of wild-type membranes to all concentrations of LPA was 1695 ± 16 CPM and did not differ significantly from the basal response of 1714 ± 46 CPM, indicating no LPA-induced Gα activation and supporting other evidence that RH7777 cells are LPA non-responsive. Additionally, PTx pre-treatment reduced LPA-induced [⁳⁵S]GTPγS binding down to RH7777 wild-type levels with a mean response of 1284 ± 15 CPM, which did not differ significantly from basal, indicating that Gα mediates essentially all LPA-induced [⁳⁵S]GTPγS binding in this cell-type.

4.2.3. **Comparative effects of LPA species and lipids with reported agonist and antagonist activity at LPA receptors on [³⁵S]GTPγS binding in RH7777 membranes**

Since there are many naturally occurring forms of LPA, including various chain lengths and degrees of saturation (Baker et al., 2000) which may be able to differently activate various LPA receptors (Yoshida et al., 2003), the potency of a range of LPAs at the LPA₁ receptor was tested (Figure 4.7). The LPA variants tested exhibited a range of potencies at RH7777-LPA₁ membranes. While there was no obvious pattern for the preferred variant, the results are consistent with previous findings that suggest that compounds with a 16:0 or 18:1 fatty acyl tail are the most efficacious.
**A. DMSO tolerance**

![DMSO tolerance graph](image)

**B. Ethanol tolerance**

![Ethanol tolerance graph](image)

**Figure 4.5**

DMSO and ethanol tolerances of the [³⁵S]GTPγS binding assay. LPA concentration-response curves for [³⁵S]GTPγS binding assays performed in the presence of various final assay concentrations of DMSO (A), or ethanol (B). EC₅₀ values or window sizes did not differ statistically significantly under any observed experimental conditions.
Figure 4.6

Effect of pertussis toxin (PTx) pre-treatment on LPA responses of RH7777-LPA₁ (LPA₁) and RH7777 wild-type (WT) membranes. Untreated or PTx pre-treated RH7777-LPA₁ membranes and untreated RH7777 wild-type membranes were stimulated with the various LPA concentrations indicated. Basal levels of [³⁵S]GTPγS binding were 5730 ± 620, 1237 ± 178 and 1668 ± 181 CPM for untreated LPA₁ membranes, LPA₁ membranes treated with PTx and wild-type membranes, respectively.
Figure 4.7

$[^{35}S]$GTPγS binding stimulated by various LPA species in RH7777-LPA$_1$ membranes. **A** and **B**, concentration-response curves generated by stimulating RH7777-LPA$_1$ membranes with various LPA species as indicated. **C**, pEC$_{50}$ values obtained from the concentration-response curves shown in **A** and **B**.
The potency of the ether-linked LPAs (denoted with a ‘C’ e.g. C18:0) differed little from the ester-linked analogue, which is again consistent with previous findings that the LPA receptors are tolerant to change in the linking region between the phosphate group and fatty acyl tail (Santos et al., 2000).

Several lipids have been assessed as agonists or antagonists at RH7777-LPA$_1$ membranes using the optimised $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ assay (Figure 4.8). As has been found previously, VPC 31143 ($R$) and VPC 31144 ($S$) act as agonists, with the $R$-enantiomer having the higher potency (Heise et al., 2001). (2$S$)-OMPT also acted as a low potency agonist, though it has previously been reported to be LPA$_3$-selective (Hasegawa et al., 2003). VPC 32183, VPC 12249 and VPC 32179 were all found to act as antagonists when applied to RH7777-LPA$_1$ membranes co-stimulated with 10 nM LPA, consistent with previous reports on these compounds (Heasley et al., 2004; Heise et al., 2001). As previously reported, the fatty acid phosphate, DDP, acted as a weak antagonist (Virag et al., 2003).

There are several conflicting reports on the action of the lipid phosphoric acids, $N$-palmitoyl-$L$-serine-PA and $N$-palmitoyl-$L$-tyrosine-PA. While some studies report them to be antagonists (Liliom et al., 1996) others report agonist activity (An et al., 1998). Here, it was found that they elicited no response when applied alone as agonists or, in combination with LPA, to assess their antagonist activity.

### 4.2.4. Comparative effects of LPA species and ligands with reported agonist and antagonist activity at LPA receptors on Ca$^{2+}$ responses in SW982 cells and transfected Chem-1 cells

Following investigation of the effects of stimulation with different LPA species and LPA receptor compounds by $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding in RH7777-LPA$_1$ membranes, the study was broadened to include other LPA receptors. This was undertaken using Chem-1 cell-lines stably expressing human LPA receptor subtypes and SW982 cells, human synovial sarcoma-derived fibroblasts that are LPA-responsive. A high-throughput Ca$^{2+}$ assay which measures fluorescence changes of the Ca$^{2+}$-indicator dye Calcium-4 in a cell population was used. As with the $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ assay on RH7777-LPA$_1$ membranes, the LPAs tested exhibited a range of potencies at the cell-lines tested (Figure 4.9 and Figure 4.10). The responses of cells to the applied lipids were strikingly similar and, for most lipids, EC$_{50}$ values did not vary significantly across the cell-lines tested. The exceptions being that LPA 20:4 and VPC 31143 were significantly more potent at
Figure 4.8

Agonist and antagonist effects of commercially-available lipids on [\(^{35}\)S]GTP\(_{\gamma}\)S binding in RH7777-LPA\(_1\) membranes. Concentration-response curves generated by stimulating RH7777-LPA\(_1\) membranes with various lipids, where an agonist response was (A) or was not (B) observed. Concentration-response curves generated by stimulating RH7777-LPA\(_1\) membranes with various lipids in the presence of 10 nM LPA, where an antagonist response was (C) or was not (D) observed. In C and D, the LPA curve shown in black was generated in the absence of any additional LPA and the dotted line indicates the magnitude of response observed when membranes are stimulated with 10 nM LPA alone (i.e. the expected baseline for antagonist curves). E and F, pEC\(_{50}/pIC_{50}\) values observed in A and C, respectively.
I. VPC 12249

J. VPC 32179

K. VPC 32183

L. VPC 31143

M. VPC 31144

N. N-P-Ser-PA

O. N-P-Tyr-PA
Figure 4.9

Cell-population \([Ca^{2+}]_i\) responses stimulated by various LPA species and LPA receptor agonists and antagonists in SW982 cells and Chem-1 cells over-expressing various human LPA receptors. Responses for each experiment are normalised, such that 0 % represents unstimulated basal and 100 % represents the response of Chem-1-LPA₁ cells to 3 µM LPA (18:1). Each graph shows the response of each cell-line to the indicated compound. The symbol shape and colours inset in A and I are maintained for all graphs.
Figure 4.10

Bar graphs showing pEC$_{50}$ values observed from the experimental data shown in Figure 4.9. Each graph shows the pEC$_{50}$ observed for [Ca$^{2+}$]$_i$ responses in the indicated cell-line for each of the LPA species, or LPA receptor agonist or antagonist tested.
SW982 cells than any of the Chem-1 cells tested (two-way ANOVA, p ≤ 0.05) and that VPC 32179 displayed agonist activity in SW982 cells only. The robust responses observed in the Chem-1 wild-type cells were unexpected and prevented the identification of the particular LPA receptor subtypes responsible for the observed responses in the transfected cell-lines. The rank order of potencies for the LPAs tested was broadly similar to that observed for $[{^{35}}S]GTP_{iS}$ binding in RH7777-LPA$_1$ membranes, with ether- and ester-linked LPAs acting similarly and C18:0 LPA notably less potent than LPAs of other chain lengths.

The effects of the lipids with reported agonist or antagonist activity at LPA receptors were also largely consistent with the literature and the $[^{35}S]GTP_{iS}$ binding data reported here. VPC 31143 and VPC 31144 were found to act as agonists in the Ca$^{2+}$ assay, with the (R) enantiomer, VPC 31143, having the higher potency. N-P-Tyr-PA, VPC 12249 and its derivative, VPC 32183, exhibited no activity at any of the cell-lines tested. N-P-Ser-PA showed some activity at high concentrations in most cell-lines tested. While VPC 32179 showed no activity at most cell lines tested, it exhibited some agonist activity at SW982 cells.

4.2.5.  Investigation of LPA receptor expression in human and rat cell lines by RT-PCR

Since Chem-1 wild-type cells were unexpectedly responsive to LPA in a Ca$^{2+}$ assay, precluding definitive assignment of the observed responses to any particular LPA receptor subtype, LPA receptor expression was investigated using a non-quantitative RT-PCR approach. Firstly, cDNA samples generated from cell-lines used in this project were collected and tested for gDNA contamination by probing RT+ and RT- controls for 18S rRNA (Figure 4.11). All samples were found to be free from gDNA contamination and suitable for use. Primers were designed for human and rat LPAR1-6/Lpar1-6 and their specificity was assessed using plasmids containing the coding DNA sequence (CDS) for each receptor gene, though note that these were unavailable for rLpar4-6. All primers successfully detected their intended target only, and none of the other targets tested, with the exception of hLPAR2 primers, which also detect rLpar2 at a low efficiency (Figure 4.12).
Figure 4.11

Agarose gels showing DNA fragments produced by RT-PCR amplification of 18S rRNA using cDNA templates generated from total RNA of various cell-lines as indicated. Plus and minus signs indicate presence or absence of enzyme in the reverse transcription. Water was used in place of template in the PCR for a negative control. Numbers on the left indicate size of DNA ladder fragments in bp. Arrows show expected PCR product band size which is 200 bp.
Figure 4.12
Agarose gels showing DNA fragments produced by PCR amplification of human LPAR1-6 and rat Lpar1-3 sequences using plasmid DNA containing the indicated LPA receptor gene sequences as template, in order to assess primer specificity. Water was used in place of template in the PCR for a negative control. Numbers on the left indicate size of DNA ladder fragments in bp. Arrows show expected band sizes of PCR product which are 570, 286, 140, 360, 238 and 349 bp for hLPAR1-6, respectively, and 648, 175 and 135 bp for rLpar1-3, respectively.
Probing the cDNA samples with primers designed for each of the rat and human LPA receptor genes showed that the Chem-1 cells endogenously express rat LPA receptors at the mRNA level; *tLpar*-2, -5 and -6 were detected in all Chem-1 cDNA samples studied (Figure 4.13 and Table 4.1). Probing Chem-1 cDNA samples with primers for the human receptors showed that the stably transfected LPA₁ and LPA₃ cell-lines expressed the appropriate human LPA receptor, but no h*LPAR*₂ was detected in the LPA₂ stable cell-line (Figure 4.14 and Table 4.1), suggesting that transfection had been unsuccessful, or that expression had been lost during the initial passaging of this cell-line. The human-derived SW982 cell-line and primary cultured human lung fibroblasts and hASM cells were also tested. These were all found to share the same LPA receptor expression profile, with h*LPAR*-1, -2, -3 and -6 all being detected (Figure 4.14 and Table 4.1).
Figure 4.13

Agarose gels showing DNA fragments produced by RT-PCR amplification of rat Lpar1-6 gene sequences using cDNA templates generated from total RNA of indicated cell lines. Plus and minus signs signify presence or absence of enzyme in the reverse transcription. Water was used in place of template in the PCR for a negative control. Plasmids containing the CDS of the appropriate rLpar gene were used as PCR template for a positive control for rLpar1-3. For rLpar4-6, cDNA generated from total RNA of whole rat brain homogenate was used as PCR template for a positive control. Numbers on the left show size of DNA ladder fragments in bp. Arrows indicate expected band sizes of PCR products; 648, 175, 135, 390, 278 and 434 bp for rLpar1-6, respectively.
Figure 4.14

Agarose gels showing DNA fragments produced by RT-PCR amplification of human LPAR1-6 genes using cDNA templates generated from total RNA of indicated cell-lines. Plus and minus signs signify presence or absence of enzyme in the reverse transcription. Water was used in place of template in the PCR for a negative control. Plasmids containing the CDS of the appropriate LPAR gene sequence were used as PCR template for a positive control. Numbers on the left show size of DNA ladder fragments in bp. Arrows indicate expected band sizes of PCR products; 570, 286, 140, 360, 238 and 349 bp for hLPAR1-6, respectively.
Table 4.1

Table summarising results of RT-PCR experiments investigating LPA receptor expression in various cell-lines used. Plus and minus signs denote the presence or absence of a band when probing with primers for the indicated rat or human receptor. HLF, human lung fibroblasts; hASM, human airway smooth muscle cells.
4.3. Discussion

In this Chapter, I aimed to better characterise the effects of several lipids on LPA receptor activity, including a range of endogenous LPA species and a series of lipids with reported agonist or antagonist activity at LPA receptors.

RH7777 cells have emerged as one of the preferred model cell-lines for investigation of recombinant LPA receptors, as while the LPA receptors are almost ubiquitously expressed, LPA elicits no response in a broad range of assay readouts in these cells, including $[^{35}\text{S}]$GTP$_{\gamma}$S binding, specific $[^{3}\text{H}]$LPA binding, neurite retraction, membrane blebbing, cyclic AMP accumulation and SRE-luciferase reporter assays (Fukushima et al., 1998; Yanagida et al., 2009). This enables characterisation of recombinant LPA receptors using $[^{35}\text{S}]$GTP$_{\gamma}$S binding within a response-null background. It should be noted that northern blotting analysis indicates that RH7777 cells may express the recently identified LPA$_5$ receptor (Lee et al., 2006), although functional responses upon LPA stimulation have not been reported.

A total $[^{35}\text{S}]$GTP$_{\gamma}$S binding assay has been optimised for measuring LPA$_1$ receptor activation in the RH7777-LPA$_1$ cell-line. This assay is widely used to measure G protein activation in response to receptor stimulation, since it allows measurement of an early signal transduction event proximal to receptor-ligand binding and before amplification and diversification, as might occur for more distal signalling events. The assay was optimised for use in this system with respect to incubation time and assay component concentrations. Required conditions were found to be typical for the assay, including a relatively high GDP concentration (3 µM) to drive down basal levels of guanine nucleotide exchange, 300 pM $[^{35}\text{S}]$GTP$_{\gamma}$S and 20 µg/well membrane protein to achieve adequate levels of $[^{35}\text{S}]$GTP$_{\gamma}$S binding. The observed basal levels of activation increased with time without affecting measured agonist potency, such that the observed window, or dynamic range, of activation decreased with incubation time. For this reason, incubation times were kept short (30 min), with an additional pre-incubation of 30 min to allow for antagonist binding where appropriate. Experiments were performed to ensure that LPA was not being broken down chemically or biologically during the assay. LPA is known to be broken down by the lipid phosphate phosphatase, LPP1 (Pilquil et al., 2001). Use of the broad spectrum phosphatase inhibitor sodium
orthovanadate in the assay offered no benefit, indicating that phosphatases are not causing any significant LPA breakdown during the assay. Preparations of LPA left at room temperature for 24 hours were found to be equipotent with freshly prepared LPA, indicating that no significant chemical breakdown had occurred.

Since a wide variety of cell-types are known to be LPA-responsive it was important to test RH7777 wild-type cells for any LPA response in the $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding assay. As has been previously reported in the literature (Fukushima et al., 1998; Lee et al., 2006), LPA elicited no increase in total $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding in RH7777 wild-type cells up to a concentration of 3 µM, indicating this to be a suitable cell-line for pharmacological characterisation of the LPA receptors using this assay. Since the $\text{G}_\alpha_{i/o}$ family of G proteins are typically expressed at a much higher level and have an apparently higher basal rate of guanine nucleotide exchange than other $\text{G}_\alpha$ subtypes, the basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding attributable to $\text{G}_\alpha_{i/o}-[^{35}\text{S}]\text{GTP} \gamma \text{S}$ complexes may mask the contribution of other $\text{G}_\alpha$ subtypes to the total $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding (Milligan, 2003). To assess the contribution of $\text{G}_\alpha_{i/o}-[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding to the total response observed, the effect of PTx pre-treatment of RH7777-LPA$_1$ cells on derived membrane preparations was investigated. PTx pre-treatment completely eliminated total $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding in RH7777-LPA$_1$ cell membranes to levels observed in membranes prepared from RH7777 wild-type cells, indicating that other $\text{G}_\alpha$ subtypes are likely to make at most, a minor contribution to the total receptor-activated $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding. However, RH7777 cells are known to express a range of $\text{G}_\alpha$ subtypes and LPA$_1$ is proposed to couple to members of the $\text{G}_\alpha_{i/o}$, $\text{G}_\alpha_{q/11}$ and $\text{G}_\alpha_{12/13}$ families (Fukushima et al., 1998), so it is likely that at least some contribution is made by these other subtypes, even if it is undetectable under the conditions used here, which will have been optimised towards detection of $\text{G}_\alpha_{i/o}-[^{35}\text{S}]\text{GTP} \gamma \text{S}$ complexes.

It is interesting to note that expression of the LPA$_1$ receptor causes a marked increase in the basal level of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding; with the basal response increasing more than three-fold (from 1714 ± 46 to 5913 ± 68 CPM; Figure 4.6) with receptor expression. There are two potential explanations for this: firstly and most simply, some contaminating LPA in the membrane preparation is causing LPA receptor activation so that the basal level of binding being observed for the RH7777-LPA$_1$ membranes is not a true basal; secondly, and potentially more interestingly, the increase could be caused by
constitutive activity of the LPA₁ receptor that is causing significant ‘basal’ G protein activation. Liquid chromatography/mass spectrometry has shown that LPA is found at micromolar concentrations in human serum (Baker et al., 2001), indeed LPA is thought to contribute significantly to the proliferative and protective effects of serum in cell culture (Chen et al., 2008), so there would be a relatively high concentration of LPA present during the culture of RH7777-LPA₁ cells. Although the washing steps involved in the membrane preparation were rigorous, the possibility of contaminating LPA remains. LPA is also likely to be present in small quantities in the sample since it is a minor membrane constituent, though whether this LPA is biologically available to bind receptor remains a matter for debate. Additionally, expression of the enzymes responsible for LPA synthesis is widespread (Giganti et al., 2008) so it is possible that LPA can be generated by these cells in culture, even if LPA is excluded as far as possible from the growth medium. Stimulation of LPA receptors by locally synthesised LPA has been reported to account for much of the autoradiographically observed basal [³⁵S]GTPγS binding in rat brain slices (Aaltonen et al., 2008), though again any locally generated LPA and LPA-producing enzyme should be removed by the washing steps in the membrane preparation. Though some groups suggest that LPA receptors do have a level of constitutive activity (Moughal et al., 2006), it is difficult to distinguish between the two possibilities since it is impossible to say whether sub-micromolar amounts of LPA remain in the sample that could be causing a partial activation of the receptor and masking the true basal response. Further study is required to elucidate the precise cause of this high basal [³⁵S]GTPγS binding.

Since there have been reports that various LPA species have different potencies at the different LPA receptors (Bandoh et al., 2000), the optimised [³⁵S]GTPγS binding assay was used to compare the effects of LPAs with varying chain lengths, degrees of saturation and ester/ether linker in RH7777-LPA₁-derived membranes. It was found that modifying the chain length did indeed alter LPA potencies, but that all forms were able to stimulate increases in [³⁵S]GTPγS binding. In agreement with preliminary structure-function studies on the LPA receptors (Santos et al., 2000), it was found that the linker region between the fatty acid and glycerol backbone was quite tolerant to change as there was no significant difference between LPAs with ether rather than ester linkers and that 16:0 and 18:1 fatty acyl chains produced the most potent compounds. Since these LPAs have only yet been tested with one LPA receptor subtype using the
developed $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding assay, it is clearly not yet possible to say whether there are any differences in their relative potencies at different LPA receptor subtypes.

In order to better characterise some of the commercially-available LPA receptor-active compounds that have been reported to act as LPA receptor agonists or antagonists, the $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding assay was used to compare the effects of DDP, $N$-palmitoyl-$L$-serine-PA, $N$-palmitoyl-$L$-tyrosine-PA, VPC 31143, VPC 31144, VPC 12249, VPC 32183, VPC 32179 and (2$S$)-OMPT using RH7777-LPA$_1$ membranes. The results obtained here largely agree with previously reported findings. VPC 31143 and its $S$-enantiomer VPC 31144 acted as agonists, with the $R$-enantiomer having a greater potency than LPA (18:1) itself, as has been previously reported for $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding studies using HEK293 cells transfected with the Edg family LPA receptors (Heise et al., 2001). In the same study VPC 12249 was designated an LPA$_{1/3}$-selective antagonist (Heise et al., 2001). It has also been found that VPC 12249 lacked agonistic activity when applied alone, but when applied in the presence of an approximate EC$_{50}$ concentration of LPA, was able to inhibit the response in RH7777-LPA$_1$ membranes. Structurally similar compounds VPC 32179 and its $S$-enantiomer VPC 32183 have been reported to be more potent than their parent compound VPC 12249 and to be LPA$_{1/3}$-selective (Heasley et al., 2004). Again the results were consistent with previously published findings as VPC 32179 and VPC 32183 both inhibited the LPA response and were more potent than VPC 12249. Similar to VPC 31143 and VPC 31144, the $R$-enantiomer was more effective, which is as expected since the $R$ form is the natural conformation. Interestingly, VPC 32183 inhibited the LPA response to levels well below basal, possibly indicating inverse agonism, but again this may be due to endogenous LPA contamination as previously discussed. (2$S$)-OMPT is reported to act as an LPA$_{3}$-selective agonist (Hasegawa et al., 2003). Results presented here show that (2$S$)-OMPT can cause an increase in $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding in RH7777-LPA$_1$ membranes, albeit with low potency. The fatty acid phosphate DDP has been reported to act as an LPA$_{1/3}$-selective competitive antagonist (Virag et al., 2003) and it was also found that this compound was able to inhibit LPA-induced $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding in RH7777-LPA$_1$ membranes.

While there have been numerous reports on the effects of $N$-palmitoyl-$L$-serine-PA and $N$-palmitoyl-$L$-tyrosine-PA on LPA receptor activity, no consensus has been reached
regarding the pharmacological properties of these related compounds. Initial studies indicated that they antagonise the Cl⁻ response in *Xenopus* oocytes to endogenous LPA receptor activation (Liliom *et al.*, 1996; Bittman *et al.*, 1996), but it was then revealed that these compounds induced Ca²⁺ responses in a human breast cancer cell-line (Hooks *et al.*, 1998) and human osteoblasts (Jan *et al.*, 2003). All reported studies on these compounds have been done on endogenous receptor responses, so it has not been possible to infer which receptor subtype, if any, is responsible. Here, no response above basal and no inhibition of LPA-evoked responses to these compounds were observed in RH7777-LPA₁ membranes. This suggests that inhibition of the LPA-induced Cl⁻ current and Ca²⁺ responses induced by these compounds are not likely to be mediated by G proteins coupled to LPA₁.

Following investigation of the action of these lipids at LPA₁ containing membranes using a [³⁵S]GTPγS binding assay, it was intended that this study be broadened to include other LPA receptor subtypes to clarify the receptor subtype specificity of any effects observed. Since a robust assay for LPA receptor activity had been developed, it was intended that this work be continued in this same system, however generation of stable cell-lines expressing other LPA receptors proved to be technically challenging.

Instead, this work was continued in Chem-1 cells stably transfected to express the LPA₁, LPA₂ or LPA₃ receptor subtype purchased from Millipore. Chem-1 cells over-express Ga₁₅, allowing most GPCR subtypes to produce robust Ca²⁺ responses upon agonist challenge, enabling testing of the lipids at these three LPA receptor subtypes using a high throughput Ca²⁺ assay.

It was thought that it would be more efficient to develop the Chem-1/Ca²⁺ assay system to perform the lipid screen than to develop new cell-lines to enable continuation of the work using the [³⁵S]GTPγS binding assay. However, preliminary experiments using the Chem-1 cell system showed that the Chem-1 wild-type cells were LPA-responsive (Figure 4.9). In fact, the responses observed in wild-type cells were statistically indistinguishable from those of the LPA receptor-transfected cell-lines. This precluded the attribution of any observed response to a particular LPA receptor subtype, one of the key aims of this project. Data provided by the manufacturer of these cell-lines show that LPA (18:1) elicits no Ca²⁺ response in Chem-1 wild-type cells, though no such data
is presented in any peer-reviewed literature and specific details of the methods employed are unavailable.

It was thought likely that Chem-1 cells endogenously express one or more LPA receptor subtype which mediates the observed responses and therefore LPA receptor expression was investigated using an RT-PCR approach. Primers were designed which could distinguish between rat and human LPA receptor sequences. Testing the primers for specificity using plasmids containing the CDS for hLPAR1-6 or rLpar1-3 showed that the primers could detect, and were specific for, their intended targets. It is worth noting that primers for hLPAR2 also detect a faint band when used to probe the rLpar2 containing plasmid, though when testing cell-line-derived cDNA samples, no human LPAR2 was detected in rat samples and vice versa, so this is of little consequence here. Plasmids containing rLpar4-6 were unavailable for these experiments so the specificity of the rLpar4-6 primers could not be tested so thoroughly. However, primers for these receptors did not detect any bands when used to probe any plasmids tested (data not shown). Since the human counterparts for the rLpar4-6 sequences and other rat receptors in the Lpar family that were tested are likely to be highly similar sequences to the intended target, it is reasonable to conclude that these primers are suitably specific for use, especially since probing cDNA derived from a rat brain homogenate produced bands of the anticipated size. Reassuringly, no rat receptor sequences were detected in any human cDNA samples tested and similarly human receptor sequences were only detected in the rat cDNA samples where the y had been introduced by transfection, further evidencing the specificity of the primers used.

Probing of cDNA samples derived from the cell-lines used with the developed primers detected rLpar-2, -5 and -6 in all Chem-1 cell-lines tested. It is likely that stimulation of these receptors mediate the observed LPA-induced Ca\(^{2+}\) responses, though it is not possible to verify this as no specific agonists or antagonists are available for these receptors. While hLPAR-1 and -3 sequences are detected in the appropriate transfected Chem-1 cell-lines, there was no detection of hLPAR2 in the Chem-1-LPA\(_2\) transfected cell-line. This indicated that generation of this stable cell-line was unsuccessful, or that expression of the transfected LPA\(_2\) receptor has been lost. It is possible that the LPA-induced responses in the Chem-1 wild-type background were masking this loss of activity during development of the Chem-1-LPA\(_2\) cell-line.
Since cDNA samples derived from primary cultured human lung fibroblasts, hASM cells and the human-derived continuous SW982 cell-line were available at the time of these experiments, these were also probed for LPA receptor expression. Expression of hLPAR-1, -2, -3 and -6 was detected in all human cells tested, which is in agreement with previously reported findings that show that LPA receptors have a broad expression profile (see Introduction). While there are some reports regarding the effects of LPA treatment on hASM contractility (Sakai et al., 2003), proliferation (Ediger and Toews, 2001) and growth factor activity (Ediger et al., 2002), there are no published data regarding LPA receptor expression in this cell-type so these data represent novel findings. Interest in LPA receptor activity in lung fibroblasts has developed since the report that LPA₁ mediates the pathological recruitment of fibroblasts to the lung in idiopathic pulmonary fibrosis (IPF; Tager et al., 2008). RT-qPCR results reported in this paper showed that LPA₁ was the most highly expressed LPA receptor in lung fibroblasts in both a mouse model of IPF and an individual with IPF. Mice deficient in LPA₁ did not display the LPA-induced chemotactic response observed in fibroblasts from wild-type mice, suggesting that LPA₁ is the functionally dominant receptor subtype in fibroblasts. Another group investigated LPA receptor expression in a human lung fibroblast cell-line, CCL-151, by non-quantitative RT-PCR, similar to that performed here, and detected message for all receptor subtypes 1-6 (Shiomi et al., 2011). The data presented here offer no clues regarding LPA receptor function nor the relative levels of receptor expression, so these data are not at odds with what has previously been reported.

4.3.1. Concluding remarks and future directions

The aim of this Chapter was to provide a better characterisation of the effects of different LPA species and various commercially available lipids with reported activity at LPA receptors, and resolve some of the ambiguity in current literature regarding their action at a range of LPA receptors. A robust [³⁵S]GTPγS binding assay was developed and effectively used to investigate lipid activities at LPA₁ receptors in an LPA non-responsive RH7777 cell background. Difficulty in generation of further RH7777 cell-lines hampered continuation of this work using the established assay so work was continued using stable Chem-1 cell-lines that over-express LPA receptor subtypes. These cells are optimised for easy measurement of LPA receptor activity using a Ca²⁺
readout as they also over-express $G\alpha_{15}$ which acts to 'force' coupling to the $Ca^{2+}$
pathway (Offermanns and Simon, 1995). This was an appealing prospect since the LPA
receptors are known to couple to a broad range of $G$ proteins and signalling pathways
(see Introduction). The potential for this system to by-pass the selectivity of receptor-
$G$ protein interaction would provide a universal system for assessment of receptor
pharmacological profiles, as well as an efficient means to assay receptor activity. Unfort-
nately, the LPA-responsiveness of Chem-1-wild-type cells, together with the
lack of receptor subtype-specific antagonists, precluded characterisation of subtypes
beyond the LPA$_1$ receptor in this work. However, it has been clearly demonstrated that
a [$^{35}$S]GTP$\gamma$S binding assay can be employed very effectively to these ends. With more
time, further attempts would be made to establish RH7777 cell-lines expressing other
LPA receptor subtypes and to develop the assay for application to these cell-lines.
Completion of this work would provide some much-needed clarity regarding the
commercially available repertoire of LPA receptor-active compounds.

In this study it was found that expression of LPA$_1$ in RH7777 membranes causes a high
basal level of [$^{35}$S]GTP$\gamma$S binding, which is possibly caused by constitutive activity in
the LPA receptor. This could be further investigated by attempting to measure LPA
content in the membrane preparation. While this can neither prove nor disprove the
occurrence of constitutive activity, it would help ascertain whether there is a
pharmacologically significant LPA contamination of the membrane preparation. A
complementary approach could be to attempt to reduce the potential contamination in
the membrane samples. The only commercially available inhibitor for autotaxin, the
key enzyme involved in LPA synthesis, also acts as a pan-LPA receptor inhibitor
(Zhang et al., 2009), so would be inappropriate to use for diminishing LPA
concentration. It may be possible to transfect RH7777-LPA$_1$ cells to over-express
LPP1, a key enzyme involved in LPA degradation, to destroy any locally produced
LPA. Since LPP1 is an integral membrane protein (Waggoner et al., 1999) it would not
be washed away during the membrane preparation and so should remain active during
the assay. However, LPP1 over-expression could potentially cause the breakdown of
exogenously applied LPA, and perhaps other LPA receptor-active compounds, so
assays would need to be configured to take this complicating factor into account.
CHAPTER 5. Investigation of LPA mediated signalling in cultured human airway smooth muscle cells and other cell-types
5.1. Introduction

As discussed in the main Introduction, LPA has been shown to elicit a broad array of effects in a range of cell types. The aim of this Chapter is to investigate LPA-mediated signalling, particularly in cultured hASM cells from clinically characterised donors with and without an asthma diagnosis. The SW982 cell-line was also employed as a model to help accommodate the limited availability of hASM cells. SW982 cells are human synovial sarcoma-derived fibroblasts which are LPA-responsive and express the LPA1 receptor subtype (personal communication, M.R. Dowling). cAMP and migratory responses are the focus of this Chapter; any effects of LPA treatment on such responses in hASM cells have potential clinical significance, firstly, because these are pathophysiologically relevant functions (see below) and secondly, because LPA may be present at increased concentrations in allergic airway inflammation (Georas et al., 2007).

Initial experiments focussed on investigating effects of LPA treatment on cAMP signalling in hASM cells by measuring cAMP accumulation in cell monolayers using a cAMP/[^3]H]cAMP binding protein method (Brown et al., 1971). Several LPA receptors, including LPA1, LPA2, and LPA3 are reported to reduce cellular cAMP concentration by coupling to Ga proteins and inhibiting adenylyl cyclase (AC) activity (Ishii et al., 2000), while stimulation of LPA4 and LPA5 leads to an increase in cAMP levels by coupling to Ga and activating AC (Lee et al., 2007; Lee et al., 2006). cAMP is a pivotal regulator of many hASM cell functions, including contraction (Oguma et al., 2006), proliferation (Stewart et al., 1999), migration (Goncharova et al., 2003) and synthetic function (Ammit et al., 2000), and the pertinence of cAMP regulation of hASM cell function in the context of asthma is evidenced by the fact that increasing cellular cAMP by stimulation of β2-adrenoceptors forms the cornerstone for asthma therapy.

Studies using cultured hASM cells and isolated tracheal rings have found that LPA can affect cAMP production (Nogami et al., 1995) and smooth muscle relaxation (Toews et al., 1997; Sakai et al., 2003), arguably the most therapeutically important function of cAMP in the lung. The effects of LPA stimulation on hASM cAMP production are complex; LPA was identified as the component of serum which "sensitised" cAMP
responses, whereby LPA elicits no cAMP accumulation itself, but pre-treatment of cells with LPA substantially enhances cAMP responses induced by subsequent treatment with forskolin, isoprenaline or prostaglandin E1, which stimulate cAMP production through direct AC activation, β2-adrenoceptors and prostaglandin (EP) receptors, respectively (Kreps et al., 1993). Further investigation of this phenomenon in cultured hASM cells revealed that while pre-treatment with LPA sensitised cAMP responses, applying LPA at the time of agonist treatment has markedly different effects, reducing forskolin responses, but still augmenting β2-adrenoceptor-mediated responses (Nogami et al., 1995). In addition to this study which directly investigated the effects of LPA treatment on smooth muscle cell cAMP levels, there is evidence that cAMP-elevating agents can modify some LPA-mediated signalling in smooth muscle cells; for example, LPA induces proliferation of rat aortic vascular smooth muscle (VSM) cells and this effect can be inhibited by elevating cAMP levels with forskolin or phosphodiesterase (PDE) inhibitor addition (Adolfsson et al., 2002). Conversely, LPA treatment is able to modify the effects of cAMP-elevating agents in smooth muscle cells; Toews et al. (1997) examined the effects of LPA treatment on the contractility of smooth muscle rings isolated from cat and rabbit trachea. Tracheal rings were pre-contracted with KCl or methacholine and relaxation was induced by treatment with the cAMP-elevating agents, isoprenaline or forskolin. Treatment with LPA was found to inhibit this relaxation and to augment the methacholine-induced contraction (Toews et al., 1997). Another study showed that LPA could augment ATP-induced contraction of cultured bovine tracheal smooth muscle cells in a collagen gel contraction assay and this effect was inhibited by PDE inhibitors, forskolin or dibutyryl-cAMP, a cell-permeant cAMP mimetic (Sakai et al., 2003). Additionally, several LPA-mediated effects have been found to be pertussis toxin (PTx) sensitive, indicating that LPA is mediating its effects through Goi activation. For example, there are several reports of LPA-induced proliferation of cultured rat aortic and human VSM cells, which is sensitive to PTx treatment, while others report LPA-induced activation of MAPK or PKC in smooth muscle cells, which is PTx-sensitive (Seewald et al., 1997; Seewald et al., 1999; Cui et al., 2003; Komachi et al., 2009). Clearly, LPA can affect the signalling and function of smooth muscle cells, and cAMP seems to mediate or modulate at least a proportion of these effects.
In addition to investigating LPA-mediated modulation of cAMP production in cultured hASM cells from control and asthmatic donors, attempts were made to characterise the effects of LPA on hASM cell migration. Activation of cell migration is a well studied function of LPA receptor activation, which has been observed in a variety of cell-types. Most investigations of LPA- and LPA receptor-mediated migration have used cancer cells and other cancer models, since LPA has been proposed to promote migration and invasion in a variety of cancers (Ren et al., 2006; Zhang et al., 2009; Okumura et al., 2011); ovarian cancer has been a point of particular focus (Li et al., 2009). However, varied roles for LPA- and LPA receptor-mediated migration have been reported including a role in the pathological recruitment of fibroblasts in lung and renal fibrosis (Tager et al., 2008; Pradere et al., 2008), dental pulp cell migration in tooth repair (Cheng et al., 2010), and recruitment of mesenchymal stem cells to inflamed joints in rheumatoid arthritis (Song et al., 2010).

While there are several reports of LPA-mediated migration of rat and human VSM cells (Komachi et al., 2009; Zhou et al., 2009; Kim et al., 2006), there is only one study reporting LPA-induced migration of SM cells of the airways (Hirakawa et al., 2007). In cultured bovine smooth muscle cells isolated from trachea, LPA treatment caused increased non-directional migration, reorganisation of the cytoskeleton and RhoA translocation to the membrane. The observed migratory response was inhibited by cAMP-elevating agents, including dibutyryl-cAMP, β2-adrenoceptor agonists, forskolin and PDE inhibitors, though not when a PKA inhibitor was co-applied, indicating that PKA, activated by increased cellular cAMP levels, mitigated LPA-induced migration in bovine tracheal smooth muscle cells (Hirakawa et al., 2007).

There is an increase in airway smooth muscle mass and thickening of the airway wall that occurs as part of airway remodelling in chronic asthma (Johnson et al., 2001; Regamey et al., 2008). Many comparisons have been drawn between airway smooth muscle remodelling in asthma and the remodelling of vascular smooth muscle in atherosclerosis (Black et al., 2001; Madison, 2003). Since VSM cell migration is implicated in thickening of the blood vessel wall in atherosclerosis (Newby and Zaltsman, 2000), attention has been drawn to SM cell migration in the pathophysiology of asthma. Many studies have reported in vitro ASM cell migration in response to various stimuli, including PDGF (Carlin et al., 2003), TGF-β (Hedges et al., 1999),
fibroblast growth factor (Goncharova et al., 2003), plasminogen activators (Mukhina et al., 2000) and the chemokines CCL11 (Joubert et al., 2005), CXCL8 (Govindaraju et al., 2006) and CCL19 (Kaur et al., 2006). One study showed that hASM cells could migrate in response to stimulation with bronchial epithelial cell supernatants, demonstrating that this function of hASM occurs in response to physiologically relevant stimuli (Takeda et al., 2009). There is evidence of infiltration of myofibroblasts into the asthmatic airways, which are thought to be important contributors to airway remodelling by their deposition of ECM components and release of inflammatory and mitogenic mediators (Gizycki et al., 1997; Begueret et al., 2007). The origins of these myofibroblasts are poorly understood and one proposal is that they are derived from migrated, de-differentiated ASM cells (Bara et al., 2010). This, however, remains a controversial area of research as there is no direct in vivo evidence for increased ASM cell migration in asthma, so this investigation using hASM cells from clinically characterised donors with and without asthma can yield interesting and novel results. Here, a Boyden chamber assay (Figure 2.2) was employed to measure cell migration through a cell-permeable membrane in response to agonist treatment.
5.2. Results

5.2.1. LPA mediated regulation of cAMP production

5.2.1.1. Method optimisation and control experiments

The aim of initial experiments measuring cAMP responses of cultured hASM cells was to ascertain optimum assay conditions for measurement of LPA-mediated cAMP regulation. Time-course experiments showed that stimulation of cultured hASM cells with 10 μM forskolin caused a substantial increase in cAMP production from an unstimulated basal level of 15.7 ± 7.0 to 644.0 ± 54.1 pmol mg⁻¹ at 10 min stimulation (Figure 5.1A). Where cells had been pre-incubated with 1 μM LPA for 5 min before stimulation with forskolin, the cAMP level after forskolin stimulation for 10 min was significantly lower at 284.2 ± 39.8 pmol mg⁻¹ (two-way ANOVA, p ≤ 0.01, Bonferroni post-test). The cAMP levels observed at 10, 20 or 30 min forskolin stimulation did not differ significantly, either with or without LPA pre-incubation. It was decided that 10 min was an effective and convenient stimulation period for further experiments. A further time-course was performed to determine an effective time for LPA incubation (Figure 5.1B). Adding LPA 10 or 5 min prior to or at the same time as stimulation with forskolin inhibited the forskolin response by approximately half to 329.0 ± 49.1, 306.0 ± 34.4 and 301.0 ± 42.7 pmol mg⁻¹, respectively. There was no significant difference in cAMP response observed whether adding LPA 0-10 min prior to stimulation with forskolin. A 5 min LPA pre-incubation period was deemed convenient and was used for all further cAMP experiments.

Measurement of cAMP response is often performed in the presence of the broad-spectrum phosphodiesterase (PDE) inhibitor, IBMX to prevent cAMP degradation during the course of the assay. To investigate the need for IBMX in measuring cAMP in cultured hASM cells, the cAMP response to the non-selective β-adrenoceptor agonist, isoprenaline, was measured in the presence and absence of IBMX (500 μM) (Figure 5.2). In the absence of IBMX, isoprenaline stimulation caused a concentration dependent increase in cAMP levels from unstimulated basal levels of 19.1 ± 1.5 to 163.4 ± 40.4 pmol mg⁻¹ at 10 μM isoprenaline stimulation. However, in the presence of IBMX, isoprenaline stimulation caused a cAMP response from basal levels of 22.7 ± 6.6 to 680.5 ± 44.9 pmol mg⁻¹ at 10 μM isoprenaline stimulation. This indicates
Figure 5.1

Time-courses of cAMP production in hASM cells. A, Accumulation of cAMP over time after stimulation with forskolin (FK) (10 µM), with and without addition of LPA (1 µM) 5 min prior to FK stimulation. B, Accumulation of cAMP in response to FK (10 µM) stimulation for 10 min with and without addition of LPA (1 µM) added at the indicated times prior to FK stimulation. Compared with forskolin stimulation alone, cAMP levels are significantly lower where LPA is applied 5 or 10 minutes before, or at the same time as forskolin stimulation. Data are mean ± s.e.m. from 3 experiments using cells from 2 control donors.
Figure 5.2

Effect of IBMX addition on cAMP responses in hASM cells. Isoprenaline (iso) concentration-response curves for cAMP accumulation in hASM cells were generated in the absence or presence of IBMX (500 µM). Cells were stimulated with isoprenaline for 10 min. Data are shown as means ± s.e.m from 4 experiments using cells from 4 control donors.
approximate 8.5- and 30-fold increases in agonist-stimulated cAMP, in the absence or presence of IBMX, respectively. While the magnitude of response was significantly enhanced by IBMX treatment, the pEC50 for the isoprenaline induced cAMP response was 6.9 ± 0.3 and 7.1 ± 0.12 in the absence or presence of IBMS, respectively, and didn't differ significantly with IBMX treatment. IBMX was included in all further cAMP experiments.

There are some reports of LPA receptors coupling to Gαs proteins resulting in cAMP production (Lee et al., 2006; 2007) so LPA-induced cAMP production was investigated. Figure 5.3 shows that LPA stimulation elicited no detectable increase in cAMP levels in hASM cells. At LPA concentrations ranging from 0.1 nM to 1 µM, cAMP did not change significantly from basal unstimulated levels. Treatment of hASM cells with the LPA1/3-selective antagonist, Ki16425, had no effect on basal levels of cAMP (Figure 5.3).

5.2.1.2. LPA-mediated regulation of forskolin- or isoprenaline-induced cAMP production in cultured hASM cells from control and asthmatic donors

Several LPA receptors are known to couple to Gαi proteins to inhibit adenylyl cyclase and reduce cellular cAMP production (Ishii et al., 2004), so the effects of LPA treatment on forskolin- or isoprenaline-induced cAMP responses in hASM derived from control or asthmatic donors were investigated. Data were obtained from 6 control and 10 asthmatic donors. The magnitude of cAMP responses observed in cells derived from asthmatic patients was significantly higher than that of control donors (Figure 5.4; two-way ANOVA). On stimulation with 10 µM forskolin, cAMP levels of 644.3 ± 75.1 and 931.9 ± 184.6 pmol mg⁻¹ were observed, while stimulation with 1 µM isoprenaline resulted in cAMP levels of 504.4 ± 56.4 and 761.1 ± 115.4 pmol mg⁻¹ for control and asthmatic samples, respectively. This represents 50 % and 41 % LPA-induced decreases in the normal and asthmatic donor hASM cell FK responses, respectively.

Incubating hASM cells with LPA prior to stimulation with forskolin produced an LPA-induced, concentration-dependent decrease in cAMP levels with LPA treatment at 1 µM reducing the forskolin response to 329.4 ± 35.5 or 563.7 ± 127.7 pmol mg⁻¹ for control or asthmatics, respectively (Figure 5.5A).
Figure 5.3

Effect of LPA treatment on cAMP levels in hASM cells. hASM cell cAMP responses to forskolin (FK), Ki16425 or LPA at the indicated concentrations. Cells were stimulated for 10 min. Data are shown as means ± s.e.m from 3 experiments using cells from 3 control donors.
Figure 5.4
Comparison of cAMP responses in hASM cells from control or asthmatic donors. cAMP responses to forskolin (FK) (10 µM) or isoprenaline (iso) (1 µM) in hASM cells derived from control and asthmatic donors. Asthmatic-derived cells displayed significantly higher cAMP responses than controls when stimulating with FK or isoprenaline as determined by 2-way ANOVA with Bonferroni post-test, p ≤ 0.05. Cells were stimulated for 10 min. Data are shown as means ± s.e.m from 16 experiments using cells from 6 control donors and 10 asthmatic donors.
The effect of LPA treatment on cAMP responses to forskolin or isoprenaline in hASM cells from control and asthmatic donors. LPA concentration-response curves for cAMP accumulation in hASM cells from control and asthmatic donors. Indicated concentrations of LPA were added 5 min prior to stimulation with FK (10 μM) or isoprenaline (Iso; 1 μM) for 10 min. Data are expressed as amount of cAMP per mg of protein in the cell monolayer (A and B) or normalised to response of stimulant (FK or Iso) alone (C and D). Data are shown as means ± s.e.m. from 16 experiments using cells from 6 control donors and 10 asthmatic donors.

Figure 5.5
Treating hASM cells with LPA before stimulation with isoprenaline resulted in biphasic cAMP responses, where low LPA concentrations reduced and higher LPA concentrations increased isoprenaline-stimulated cAMP responses (Figure 5.5B). Cells derived from control donors showed basal cAMP levels of $14.7 \pm 4.5$ pmol mg$^{-1}$. Isoprenaline (1 µM) induced a response of $504.4 \pm 56.4$ pmol mg$^{-1}$ which was reduced by 28.2% to $362.0 \pm 47.8$ pmol mg$^{-1}$ at 10 nM LPA, but increased to $686.9 \pm 104.8$ pmol mg$^{-1}$ at 1 µM LPA. A similar pattern of response was observed in hASM cells from asthmatic donors, where basal cAMP concentration was $38.0 \pm 11.5$ pmol mg$^{-1}$. Isoprenaline (1 µM) induced a response of $761.1 \pm 115.4$ which was reduced by 21.3% to $598.7 \pm 130.6$ pmol mg$^{-1}$ at 10 nM LPA, which then increased to $966.0 \pm 175.1$ pmol mg$^{-1}$ with 1 µM LPA.

Normalising the LPA modulation of isoprenaline- or forskolin-induced cAMP response to the respective response to the stimulant alone, revealed that despite the difference in magnitude of responses, there was no disease-dependent difference in the sensitivity to LPA, or the patterns of response observed (Figure 5.5C and D).

Figure 5.6 shows that treatment with the LPA$_{1/3}$-selective inhibitor, Ki16425 (10 µM), reversed the effects of treatment with a near-maximal concentration of LPA (1µM), indicating that the effects observed in each case are LPA receptor-mediated.

5.2.1.3. **LPA-mediated regulation of forskolin- or isoprenaline-induced cAMP production in SW982 cells**

Since there was limited availability of primary hASM cells, which can be passaged a limited number of times, study of LPA modulation of cAMP was continued in a human continuous cell-line, SW982, which endogenously expresses LPA receptors. Initial control experiments showed that IBMX significantly enhanced observed cAMP responses to forskolin, that LPA induced no cAMP response when applied alone and that treatment with Ki16425 had no effect on basal cAMP levels, as was shown in hASM cells (Figure 5.7).

Pre-treatment of SW982 cells with LPA prior to stimulation with isoprenaline or forskolin, modulated the cAMP response in a similar fashion to that observed in hASM cells. LPA treatment caused a monophasic concentration-dependent decrease in forskolin-induced cAMP responses at a range of forskolin concentrations (Figure 5.8).
Effect of Ki16425 treatment on LPA modulation of forskolin- and isoprenaline-stimulated cAMP responses in hASM cells from control and asthmatic donors. Ki16425 was added for 10 min prior to addition of LPA which was added 5 min prior to stimulation with FK (10 µM) or isoprenaline (Iso; 1 µM) for 10 min. Data are normalised to response of the stimulant (FK or Iso). Data are mean ± s.e.m. from 16 experiments using cells from 6 control donors and 10 asthmatic donors.
Figure 5.7

A, SW982 cell-line cAMP responses to stimulation with the indicated concentrations of forskolin (FK) in the presence and absence of IBMX (500 µM). B, SW982 cell cAMP responses to FK, Ki16425 or LPA at the indicated concentrations. Cells were stimulated for 10 min for all experiments. Data are shown as means ± s.e.m. for 4 independent experiments.
Figure 5.8

LPA concentration-response curves for modulation of forskolin-induced cAMP accumulation in SW982 cells. Indicated concentrations of LPA were added 5 min prior to stimulation with indicated concentrations of forskolin (FK) for 10 min. Data are expressed as amount of cAMP per mg of protein in the cell monolayer (A) or normalised to response of stimulant (FK) alone (B). Data are shown as means ± s.e.m. for 2 independent experiments.
Observed levels of cAMP reduced from 546.9 ± 35.4 to 308.9 ± 6.4, 434.4 ± 20.3 to 195.5 ± 9.0 and 350.4 ± 4.7 to 102.8 ± 6.8 pmol mg⁻¹ with LPA (1 µM) pre-treatment prior to stimulation with 10, 3 or 1 µM forskolin, respectively (Figure 5.8A), equating to a 44.0 ± 1.2, 57.2 ± 2.2 or 74.6 ± 2.0 % reduction in forskolin-induced response with LPA (1 µM) treatment for 10, 3 or 1 µM forskolin, respectively (Figure 5.8B). The observed pIC₅₀s for LPA were 8.17 ± 0.2, 8.28 ± 0.08 and 8.34 ± 0.06 for 10, 3 or 1 µM forskolin, respectively, and did not differ significantly across the forskolin concentrations used.

Treating SW982 cells with LPA prior to stimulation with isoprenaline resulted in a biphasic cAMP response whereby lower LPA concentrations reduced, and higher LPA concentrations increased, the isoprenaline-stimulated cAMP response, as had been observed in hASM cells (Figure 5.9). Isoprenaline (1 µM) induced a cAMP accumulation of 516.9 ± 58.8 pmol mg⁻¹, which was reduced by approximately 30 % to at 3 nM LPA, then increased to 436.2 ± 62.0 pmol mg⁻¹ in the presence of isoprenaline and 1 µM LPA. In contrast to hASM cells, the increase in cAMP observed at higher LPA concentrations in SW982 cells did not exceed the cAMP response induced by isoprenaline treatment alone.

As was observed in hASM cells, treatment with the LPA₁/β-selective inhibitor, Ki16425 (10 µM), reversed the modulatory effects of treatment with a near-maximal concentration of LPA (1 µM) on both isoprenaline- and forskolin-stimulated cAMP responses (Figure 5.10).

Further experiments investigated the biphasic nature of the cAMP response observed when cells are treated with LPA in the presence of isoprenaline. Overnight pre-treatment of SW982 cells with pertussis toxin (PTx) did not significantly alter isoprenaline-stimulated cAMP accumulation, but ablated the inhibitory portion of the LPA response, and resulted in a significant potentiation of the isoprenaline-induced cAMP response, revealing the Gαᵢ-mediated aspect of the modulation (Figure 5.11A).

Removal of Ca²⁺ from the incubation medium and inclusion of the Ca²⁺ chelator EGTA (100 µM), had only a small effect on the LPA-mediated modulation of the isoprenaline cAMP response (Figure 5.11B): At 1-10 nM, LPA the extent of the inhibitory
Figure 5.9

LPA concentration-response curve for modulation of isoprenaline-induced cAMP accumulation in SW982 cells. Indicated concentrations of LPA were added 5 min prior to stimulation with isoprenaline (1 µM) for 10 min. Data are normalised to response of stimulant (Iso) alone. Data are shown as means ± s.e.m. for 5 individual experiments.
Effect of Ki16425 pre-treatment on LPA modulation of forskolin (open bars) and isoprenaline (closed bars) cAMP responses in SW982 cells. Ki16425 was added for 10 min prior to addition of LPA which was added 5 min prior to stimulation with forskolin (10 µM) or isoprenaline (1 µM) for 10 min. Data are shown as means ± s.e.m. for between 3 and 10 individual experiments.

**Figure 5.10**
Figure 5.11

Effect of PTx treatment (A) or removal of extracellular Ca\(^{2+}\) (B) on LPA concentration-response curves for modulation of isoprenaline-induced cAMP accumulation in SW982 cells. Indicated concentrations of LPA were added 5 min prior to stimulation with indicated concentrations of isoprenaline (1 µM) for 10 min. Data are normalised to response of stimulant (iso) alone. Where indicated, cells were treated overnight with PTx (100 ng mL\(^{-1}\)). Where indicated, cell monolayers were washed and equilibrated (20 min) in Ca\(^{2+}\) free buffer which included no CaCl\(_2\) and was supplemented with 100 µM EGTA prior to experimentation. Data are shown as means ± s.e.m. for 3 individual experiments.
modulation of isoprenaline-stimulated cAMP accumulation appeared to be greater in the absence of [Ca\(^{2+}\)]\(_{e}\) (Figure 5.11B), though this was statistically insignificant.

5.2.1. **Effect of LPA on cell migration**

5.2.1.1. *Method optimisation and characterisation of LPA-induced migration in SW982 cells*

Optimal assay conditions for measurement of LPA-induced cell migration using a Boyden chamber assay were investigated for SW982 cells. Figure 5.12 shows micrographs of typical assay results, illustrating that LPA treatment can induce a large increase in SW982 cell migration. Time-course experiments showed that LPA treatment (1 µM) caused a significant increase in the number of migrated cells after 6 and 8 hours (Figure 5.13A). A stimulation period of 6 hours was used for future experiments as it allowed for a practicable and effective assay. LPA-induced SW982 cell migration was found to be concentration-dependent with a pEC\(_{50}\) of 7.84 ± 0.20 (Figure 5.13B). SW982 cell migration was increased approximately 6-fold by LPA (1 µM) treatment.

Boyden chambers are sometimes used to determine directionality of cell migration by including the stimulant in the lower, upper or both chambers (see Materials and Methods, Figure 2.2 for a diagram of the Boyden chamber assay set-up). Inclusion of LPA in the upper, lower or both assay chambers caused significant increases in the number of cells that migrated from the upper to lower chambers (Figure 5.14). There was no significant difference in the LPA responses observed in the conditions tested, indicating that there was no directionality to the migration response observed. LPA was included in the lower chamber for all other experiments.

5.2.1.2. *Attempts to measure LPA-induced migration of primary hASM cells*

It was initially hoped that an assay for measurement of LPA-induced cell migration would be developed using the SW982 cell-line, which could then be translated for use with the clinically characterised, primary hASM cells available. Several attempts were made to measure hASM migration, including using cells from several donors, using a variety of time-courses, stimulating hASM cell migration using LPA or bovine serum and implementing a variety of serum-starving conditions and methods for cell
Figure 5.12

Sample micrographs of processed Transwell filters from SW982 cell migration experiments. Cells were placed in an upper chamber and the indicated stimulus added to the lower chamber. After 6 hours incubation, cells which had migrated to the underside of the permeable membrane that separates the two chambers were fixed (methanol), stained (propidium iodide, 1:2000) and mounted onto coverslips and imaged using a fluorescence microscope. Images are shown at 20 x magnification.
Figure 5.13

Time- (A) and concentration- (B) dependencies of LPA-induced migration of SW982 cells. A, LPA (1 μM) was absent (control) or present (LPA) in the lower chamber of a Transwell assay and cells were allowed to migrate for the indicated times. B, the indicated concentrations of LPA were included in the lower chamber of a Transwell assay and cells were allowed to migrate for 6 hours. Data are shown as means ± s.e.m. for between 3 and 7 individual experiments.
Figure 5.14

Directionality of LPA-induced migration of SW982 cells. LPA (1 μM) was included in the indicated chambers of a Transwell assay and cells were allowed to migrate for 6 hours. Data are shown as means ± s.e.m. for 4 individual experiments.
harvesting. In all conditions tested, essentially no hASM cell migration was observed (data not shown).

5.2.1.3. **Investigation of the underlying mechanism mediating LPA-induced migration in SW982 cells**

Some effects of treating cells with serum have been attributed to the LPA it contains (Chen *et al.*, 2008), so the migration of SW982 cells in response to treatment with LPA was compared to that of bovine serum (Figure 5.15A). Serum treatment increased SW982 cell migration approximately 2-fold from basal levels of 33.3 ± 1.5 to 74.6 ± 6.0, while LPA treatment caused an approximate 6-fold increase to 212.7 ± 15.2 migrated cells counted per field of view. Serum-induced SW982 cell migration was completely ablated by pre-treatment of cells with the LPA₁/₃-selective antagonist, Ki16425, strongly suggesting that the observed response is LPA-receptor mediated (Figure 5.15A). Antagonist pre-treatment reduced LPA-induced SW982 cell migration to a level insignificantly different to basal levels. LPA-induced SW982 cell migration was also found to be completely inhibited by PTx pre-treatment, indicating a Gαi dependency (Figure 5.15B). PTx pre-treatment appeared to reduce basal levels of cell migration, although this was found to be insignificantly altered from basal levels without PTx pre-treatment.

In order to further investigate the mechanism(s) underlying LPA-mediated cell migration, the sensitivity of this response to a range of protein kinase inhibitors was assessed (Figure 5.16). The protein kinase inhibitors employed were Y27632, an inhibitor of the Rho-associated protein kinase, ROCK1; LY294002, a phosphoinositide 3-kinase (PI3K) inhibitor; and PD0325901, an inhibitor of mitogen-activated protein kinase kinase 1 and 2 (MEK1/2). Cells were treated with inhibitors prior to and during LPA-stimulation. LPA treatment caused a significant increase in the number of migrated cells to 173.5 ± 26.0 compared with basal levels of 22.3 ± 5.7 cells, and this was significantly reduced by treatment with each of the inhibitors applied; indeed, all inhibitors reduced SW982 cell migration to a level insignificantly different to basal rates.

Since a MEK1/2 inhibitor was able to reduce the observed LPA-mediated SW982 cell migration, LPA-mediated ERK activation was investigated using a phospho-specific
A. effects of Ki16425 treatment on serum and LPA induced migration of SW982 cells. Bovine serum (10 %) or LPA (1 µM) were included in the lower chamber of a Transwell assay and cells were allowed to migrate for 6 hours. Where indicated, cells were incubated with Ki16425 (10 µM) for 15 min prior to exposure to serum or LPA. Ki16425 was present throughout the 6 hour incubation period. B, effects of PTx pre-treatment on LPA-induced SW982 cell migration. LPA (1 µM) was absent or present in the lower chamber of a transwell assay and cells (±PTx; 100 ng mL⁻¹), were allowed to migrate for 6 hours. Data are shown as means ± s.e.m. for 3 individual experiments.
Figure 5.16

Effects of treatment with a range of protein kinase inhibitors on LPA induced migration of SW982 cells. LPA (1 µM) was included in the lower chamber of a Transwell assay and cells were allowed to migrate for 6 hours. Where indicated, cells were incubated with Y27632 (10 µM), LY294002 (10 µM) or PD0325901 (1 µM) for 15 min prior to exposure to LPA. Inhibitors were present throughout the 6 hour incubation period. Data are shown as means ± s.e.m. for 4 individual experiments.
antibody and Western blotting. A significant amount of ERK-phosphorylation was observed in untreated SW982 cells, which was significantly increased following LPA addition by $2.46 \pm 0.43$ fold (Figure 5.17). Pre-treatment with Ki16425 had no effect on basal levels, but significantly reduced the LPA-induced ERK-phosphorylation observed. Pre-treatment of SW982 cells with PD0325901 completely ablated ERK-phosphorylation, in either untreated cells, or those stimulated with LPA.
Figure 5.17

LPA-induced ERK-phosphorylation in SW982 cells. **A.** mean densitometry data for ERK-phosphorylation experiments. Cells were stimulated with LPA (1 µM) in the absence or presence of Ki16425 (10 µM) or PD0325901 (1 µM) as indicated. Cell monolayers were treated with inhibitors (Ki16425 or PD0325901) for 5 min prior to LPA stimulation for 10 min before lysis. Data are shown as means ± s.e.m. for between 3 and 6 individual experiments. **B.** a representative blot of the data described in **A.**
5.3. Discussion

The aims of this Chapter were to characterise LPA-mediated signalling events and investigate any disease-dependent trends in cultured primary hASM cells. Work focussed on two signalling read-outs of potential significance to asthma pathophysiology which are known to be altered by LPA treatment; cAMP and cell migration.

5.3.1. Investigation of the effects of LPA on cAMP responses in hASM cells from control and asthmatic donors

Initial experiments intended to ascertain appropriate experimental conditions for observation of any LPA mediated effects on cellular cAMP levels. cAMP responses appeared to be stable over time, reaching a maximum after 5-10 min stimulation with forskolin (10 µM), perhaps indicating the maximum response of the system observed (Figure 5.1A). Still, LPA pre-treatment was able to significantly reduce the observed response (Figure 5.1A). The time-course of LPA treatment also appeared to be quite stable (Figure 5.1B) and a time-course of 10 min stimulation with a 5 min LPA pre-incubation was selected for further work. Inclusion of the pan PDE inhibitor, IBMX, greatly enhanced the magnitude of cAMP response observed (Figure 5.2) and was included in further work to facilitate detection of response and to minimise the potentially variable effects PDEs. While some LPA receptors have been found to couple to Gαs proteins (Lee et al., 2007; Lee et al., 2006), causing increases in cellular cAMP levels, LPA was found to elicit no cAMP response in hASM cells (Figure 5.3), indicating either a lack of Gαs coupled LPA receptor expression in hASM cells or that such coupling is not functional in the practised experimental and culture conditions.

Forskolin- and isoprenaline-induced cAMP responses were found to be significantly higher in hASM cells from asthmatic donors than controls (Figure 5.4). This result was somewhat surprising as some reports have suggested a decreased turnover of cAMP in asthma. One study in a canine model of allergic asthma reported decreased basal levels of cAMP (Rinard et al., 1979) compared to control animals, while another study using a canine model indicated that this effect may be specific to β2-adrenoceptor stimulation as it couldn’t be recapitulated with forskolin stimulation (Emala et al., 1993) as is presented in the data here. The only previously reported investigation of cAMP
accumulation in hASM cells from clinically characterised asthmatic patients showed that cells from asthmatic donors had decreased cAMP responses compared to controls, which was attributed to increased PDE4 activity (Trian et al., 2011). The disparity between the two sets of results could derive from the difference in hASM cell passage used; Trian et al. (2011) used cells between passages five and eight whereas cells in this study were cultured for no longer than four passages. There is evidence to suggest that drug treatment can alter β2-adrenoceptor activity and that prolonged exposure to β2-adrenoceptor agonists can lead to desensitisation of β2-adrenoceptors and altered PDE activity in hASM cells (Penn et al., 1998; Nino et al., 2009) which presents as a loss of therapeutic benefit from β2-adrenoceptor agonists in some asthmatics (Cheung et al., 1992). Since asthma in some of the patients used in this study was categorised as severe, it is likely that at least some were regularly exposed to β2-adrenoceptor agonists. The effects of removing hASM cells from this, other treatments and the potentially inflammatory environment of the lung to cultured conditions are uncertain. It is possible that in removing the endogenous environment through cell culture, a compensatory mechanism acting to increase cellular cAMP levels has been unveiled here.

LPA was found to modulate forskolin- and isoprenaline-induced cAMP responses in hASM cells from control and asthmatic subjects (Figure 5.5). The increased cAMP responses in cells from asthmatics is evident across all concentrations of LPA treatment, whether stimulating with forskolin or isoprenaline (Figure 5.5A and B). The consistency of the difference indicates that while the magnitude of the response is higher in the asthmatics, the effects of LPA treatment are not disease-dependent. Normalising the effect of LPA treatment to the magnitude of agonist induced cAMP response removes the difference between control and asthmatic cells completely (Figure 5.5C and D). LPA inhibited the forskolin-induced cAMP response in a concentration-dependent fashion (Figure 5.5A and C), as would be predicted by the existing literature reporting Goi coupling of several LPA receptors and the PTx sensitivity of many LPA mediated events (Tigyi et al., 1994; Zhou et al., 1995; Ishii et al., 2004). A study on the effects of LPA on hASM cell cAMP responses also found that short term LPA treatment inhibits forskolin-induced cAMP responses; this effect was found to be PTx sensitive, implicating Goi mediation (Nogami et al., 1995). As in this study, Nogami et al. (1995) also investigated the effects of LPA on isoprenaline-induced, and therefore receptor-mediated, cAMP responses. While Nogami et al. (1995) reported a
monophasic potentiation of isoprenaline-induced cAMP responses with short-term LPA treatment, we have found that LPA induced a biphasic modulation of response, whereby low concentrations of LPA inhibit, and high concentrations potentiate the isoprenaline-mediated cAMP response in hASM cells (Figure 5.5B and D).

In the present study, LPA was included in the assay for 5 min prior to 10 min of stimulation (isoprenaline or forskolin), whereas Nogami et al. (1995) either added LPA at the same time as stimulant (isoprenaline or forskolin) and incubated for 2 min or pre-incubated with LPA for 1 hour before washing cells and applying stimulant for 2 min. In both reports, acute LPA treatment produced an inhibition of forskolin response. However, Nogami et al. (1995) found that chronic LPA treatment (60 min pre-incubation) produced a potentiation of forskolin response, though no further experiments were reported to address the differing effects of chronic and acute LPA treatment. LPA is known to have diverse effects, ranging from more acute signalling events such as inhibition of cAMP production, Ca^{2+} mobilisation and RhoA activation, to more long-term effects including regulation of transcription, cell migration, cell proliferation and synthetic function (Tigyi et al., 1994; Thoreson et al., 2002; Kusama et al., 2003; Iyoda et al., 2012; Zhang et al., 2012). Further work is needed to understand the effects of chronic LPA treatment and the possible implications of this in hASM cells and asthma.

Treatment of hASM cells with the LPA_{1/3}-selective antagonist, Ki16425, for 10 min prior to LPA addition, neutralised the effects of LPA on forskolin- or isoprenaline-induced cAMP responses (Figure 5.6). At the concentration used (10 µM), Ki16425 can effectively inhibit the action of both LPA_{1} and LPA_{3} (Ohta et al., 2003). This indicates that the observed effects are LPA_{1/3}-mediated and, owing to the consistency of effect in asthmatics and controls, that there is no difference in the profile of LPA receptor activity in control and asthmatic groups. It must be noted that only the effects of a single, near-maximal concentration of LPA (1 µM) were investigated, though since Ki16425 was effective at neutralising both the inhibitory and potentiating effects of LPA on forskolin and isoprenaline induced cAMP responses, respectively, it is likely that all observed effects of LPA are indeed LPA receptor mediated.
5.3.2. **Investigation of the effects of LPA on cAMP responses in SW982 cells**

To further investigate the effects of LPA on cAMP cellular responses, the SW982 model cell line was employed, avoiding the limitations of access to primary cultures of hASM cells. As was observed in hASM cells, IBMX greatly enhanced observed cAMP responses (Figure 5.7A) and LPA elicited no cAMP response itself (Figure 5.7B). The LPA mediated modulation of forskolin- and isoprenaline-induced cAMP responses observed in hASM cells could be recapitulated in the SW982 cell background, with LPA inhibiting forskolin-induced cAMP responses in a dose dependent fashion (Figure 5.8) and having a biphasic effect on isoprenaline responses (Figure 5.9). While LPA had a biphasic on the isoprenaline response, there were some differences between these responses in SW982 cells and hASM cells. The potentiation phase of the biphasic effect did not overcome the inhibitory phase in SW982 cells, whereas in hASM cells, higher concentrations of LPA caused a large potentiation above the level of response from isoprenaline alone (Figure 5.5). As found with hASM cells, treatment of SW982 cells with the LPA₁/₃-selective antagonist, Ki16425, for 10 min prior to LPA addition, neutralised the effects of LPA on forskolin- or isoprenaline- induced cAMP responses (Figure 5.10), indicating that the observed effects are LPA₁/₃-mediated. The employed concentration of LPA (1 µM) caused an apparent inhibition of isoprenaline response, in contrast to the potentiation seen with hASM cells, due to the lower level of potentiation observed in this cell type. Across the 2 cell types studied, Ki16425 has effectively blocked LPA-mediated inhibition of forskolin responses, as well as inhibition and potentiation of isoprenaline responses, giving further weight to the notion that all LPA effects observed are LPA receptor mediated.

Using SW982 cells as a model, attempts were made to dissect the mechanism underlying the two portions of LPA's effect on isoprenaline response. Nogami *et al.* (1995) reported that while LPA's inhibitory effects on forskolin responses were PTx sensitive, that LPA-mediated potentiation of isoprenaline responses was relatively insensitive to PTx treatment, though recall that in their study, only potentiating effects of LPA on isoprenaline responses were observed. Overnight treatment of SW982 cells with PTx removed the inhibitory moiety of LPA's effects on isoprenaline treatment and caused a much larger potentiation of response, reaching 138.1 ± 4.6 % of the isoprenaline response alone at 1 µM LPA, compared with 80.5 ± 5.3 % in cells which
have undergone no PTx treatment (Figure 5.11). This firstly indicates that the inhibitory part of LPA’s effects is Gai-mediated, and secondly, that this Gaı-mediated effect suppresses the potentiating effects of LPA treatment, which are not mediated by Gaı proteins. Nogami et al. (1995) postulated that the contrasting effects of LPA on forskolin and isoprenaline responses could be explained by activity of the adenylyl cyclase 2 (AC2) isoenzyme. As a type II AC, the stimulatory action of Gaı at AC2 is known to be potentiated by Gβγ subunits (Tang and Gilman, 1991; Federman et al., 1992) so it is possible that LPA exerts its potentiating effect on isoprenaline responses by contributing Gβγ subunits on LPA receptor stimulation. In light of this, Nogami et al. (1995) proposed that the contrasting effects of LPA on forskolin and isoprenaline responses could be explained by the lack of Gaı activation when using forskolin to directly activate AC. LPA’s effects on forskolin were found to be completely PTx sensitive, while its potentiating effects on isoprenaline responses were insensitive to PTx treatment (Figure 5.11 and Nogami et al., 1995). If the potentiating effects of LPA are mediated by Gβγ subunits, these must be derived from G-protein trimers containing a Ga subunit other than Gaı. Since many LPA receptors, including LPA1 and LPA3, to which these effects have been attributed, have been found to promiscuously couple to several G-proteins, this is plausible (Ishii et al., 2004). However, since AC2 is insensitive to Gaı (Taussig et al., 1994) and some of LPA’s effects on cAMP production have been PTx sensitive, other AC isoforms must also contribute. Billington et al. (1999) report sensitisation of AC with long-term (18 hours) treatment with a number of Gaı stimulating agonists, which had inhibitory effects with short-term treatment. Such effects were found to be PTx sensitive, in contrast to the effect of acute LPA treatment on isoprenaline responses in the this study (Figure 5.11), so sensitisation by chronic agonist treatment on cAMP production (Billington et al., 1999; Nogami et al., 1995) is likely mediated by alternative mechanisms to those of the acute sensitisation observed here. There are conflicting reports on AC isoform expression in hASM cells. A recent study reported the expression of AC2, 4 and 6 in cultured human bronchial smooth muscle cells (Bogard et al., 2011), while another group reported expression of 7 AC isoforms and indicate a dominant role for AC5 (Xu et al., 2001), and another detected expression of AC2, 6, 7 and 9 and suggest a dominant role for AC6 (Billington et al., 1999). All these reports of AC expression in hASM cells relied on quantification of mRNA levels and characterisation of AC function in cells expressing multiple isoforms,
rather than quantifying AC protein directly, due to the lack of specific antibodies for AC isoforms. Expression of particular AC isoforms may account for the modulating effects of LPA on cAMP production in hASM cells, particularly since AC activity is thought to be the limiting component in hASM cAMP production (Billington et al., 1999; MacEwan et al., 1996). However, this would be difficult to verify with the currently available tools, and would be confounded by concurrent expression of multiple AC isoforms and the likely existence of cAMP micro-domains in hASM cells (Bogard et al., 2011; Willoughby and Cooper, 2007).

The activity of several AC isoforms can be modulated by Ca\(^{2+}\), calmodulin (CaM) and CaM kinases. For example, AC5 and AC6 are inhibited by free Ca\(^{2+}\) (Guillou et al., 1999), while AC1, AC3 and AC8 can be stimulated by calmodulin (Choi et al., 1992; Cali et al., 1994). Such effects are thought to specifically relate to Ca\(^{2+}\) entry, rather than receptor-mediated Ca\(^{2+}\) release from intracellular stores (Martin et al., 2009; Chiono et al., 1995; Watson et al., 2000). Depleting extracellular Ca\(^{2+}\) by omitting Ca\(^{2+}\) from the assay buffer and including the high-affinity Ca\(^{2+}\) chelator, EGTA, had no discernible effect on LPA modulation of isoprenaline-induced cAMP responses (Figure 5.11B). This may indicate involvement of an AC isoform that isn't modulated by Ca\(^{2+}\).

5.3.3. Investigation of LPA-mediated cell migration

As has been observed in a range of cell types, LPA evoked migration of SW982 cells in a time and concentration-dependent manner (Figure 5.12 and Figure 5.13). Including LPA in the upper, lower or both upper and lower chambers for stimulation caused equal levels of cell migration (Figure 5.14). While some groups have used such methods to imply directionality of migration responses and altered migration responses have been reported (Yu et al., 2009), it is unclear how a gradient of agonist can be maintained when the membrane dividing the chambers is cell permeable and so certainly agonist permeable.

LPA was found to be approximately three times as effective as serum in stimulating migration of SW982 cells and the SW982 cell migration induced by either stimulant could be completely inhibited by treatment with the LPA\(_{1,3}\)-selective antagonist, Ki16425, indicating that all observed migration is LPA receptor mediated (Figure 5.15A). LPA has been identified as a major active component of serum, accounting for
much of the pro-survival, pro-proliferative and MAPK activating effects of serum in a range of studies (Radhika et al., 2005; Chen et al., 2008; Song et al., 2005). The difference in effectiveness of serum and LPA could be explained by LPA accounting for all the migratory stimulus in serum and serum having an LPA content of less than the applied concentration of LPA (1 µM).

LPA-induced migration was found to be completely inhibited by PTx treatment, indicating that it is mediated by Gaᵢ (Figure 5.15B). This supports several other reports of PTx-sensitive LPA-induced migration in a range of cell types, including monocytes, fibroblasts and mesenchymal stem cells (Zhou et al., 1995; Song et al., 2010; Sakai et al., 1999) so this may represent a common mechanism of LPA-mediated migration. PTx treatment has implicated Gaᵢ in mediating cell migration in response to activation of a range of other GPCRs, including the PAF receptor, CXC chemokine receptors and S1P₁ (Brown et al., 2006; Thompson et al., 2007; Dutt et al., 1998; Yoon et al., 2008). Despite the implication of Gaᵢ, the low basal levels of cAMP production observed in SW982 cells (Figure 5.7) would suggest that this is not mediated by decreasing cellular cAMP levels. There is no clear consensus on the mechanism underlying LPA migration and many mediators have been implicated with Rho, members of the MAPK signalling pathway and PI3K being frequently cited (Hayashi et al., 2011; Kim et al., 2011; Sato et al., 2011; Bian et al., 2004; Panetti et al., 2000). Use of pharmacological inhibitors implicated ROCK, MAPKK and PI3K in mediating LPA-induced migration of SW982 cells (Figure 5.16).

Despite extensive testing, migration of hASM cells was not observed under any employed experimental conditions (see Results section 5.2.1.2). While this is in accordance with observations by other lab members using the same cells and culture conditions (personal communication, Prof. Chris Brightling), it contradicts some published reports of in vitro migration of airways derived smooth muscle cells (Takeda et al., 2009; Carlin et al., 2003; Mukhina et al., 2000; Joubert et al., 2005). The only reported investigation into LPA-induced migration of airway smooth muscle cells was conducted using cultured bovine cells isolated from trachea so the discrepancy may represent a species difference (Hirakawa et al., 2007). The study presented here suggests that migration is not a function of fully differentiated hASM cells and perhaps stands in support of opposing proposed mechanisms for infiltration of myo-fibroblasts.
and accumulation of hASM cell mass in asthma, for example, increased recruitment of progenitor cells.

5.3.4. **Concluding remarks and future directions**

The aim of this chapter was to characterise some aspects of LPA signalling in hASM cells, with a particular focus on disease-dependent differences between cells from control or asthmatic donors. Whilst no LPA-mediated migration of hASM cells was observed during the course of this study, hASM cells were LPA-responsive in cAMP assays, mediating its effects through LPA$_1$ or LPA$_3$ receptors, though no disease-dependent alterations in LPA receptor activity were observed. LPA exhibited complex regulation of cAMP production with PTx sensitive and insensitive pathways being employed. This could be characteristic of activation of multiple LPA receptors, the promiscuous nature of LPA receptor signalling, the complex regulation of some AC isoforms or indeed more complex mechanisms involving cross-talk between multiple receptors. There is some precedent for LPA receptor cross-talk as it has been found to alter the activity and expression of the receptor tyrosine kinase, EGFR (Cerutis *et al.*, 1997; Noguchi *et al.*, 2006). The data presented here presents more questions than answers and much further work would be required to unpick the various signalling pathways that lead to the dual effects of LPA on cAMP production observed here. Again, the insufficiency of LPA receptor subtype specific inhibitors and antibodies has been highlighted. In the absence of more selective inhibitors, siRNA knockdown of LPA receptor subtypes could help ascertain whether the dual effects of LPA are mediated by different receptor subtypes. If receptor cross-talk is implicated, LPA receptor pull-down experiments could help identify interacting partners, though the lack of antibody availability would necessitate use of exogenously expressed tagged receptors, perhaps limiting its physiological relevance. In light of the proposed role of AC isoforms in mediating the effects of LPA on cAMP production in hASM cells (Nogami *et al.*, 1995) and the emergence of the compartmentalisation of cAMP signalling into micro-domains (Willoughby and Cooper, 2007), it would be interesting to investigate the potential co-localisation of LPA receptors with individual AC isoforms, though the availability of specific antibodies against both the LPA receptors and AC isoforms is limiting here. Functional studies have pointed towards segregation of AC6 and AC2/4 signalling in hASM cells (Bogard *et al.*, 2011) and cAMP micro-
domains are likely to be an expanding field of research, particularly in smooth muscle where cAMP is such a pivotal regulator of physiological and pathophysiological function (Billington et al., 2012).

The only disease-dependent difference observed was in the magnitude of cAMP response. The increased cAMP form asthmatic derived cells stands in contrast to some reports from animal models of asthma and one study using cultured human cells (Rinard et al., 1979; Emala et al., 1993; Trian et al., 2011) and is somewhat counter-intuitive in light of the pro-relaxant and anti-inflammatory effects of increased cAMP levels (Billington et al., 2012). Perhaps this brings into question the use of a cultured cell system to measure responses which are known to be modulated by external stimulus (e.g. altered PDE or β2-adrenoceptor expression with β2-adrenoceptor agonist treatment,(Penn et al., 1998; Nino et al., 2009) ). It would be interesting to investigate cAMP responses in freshly isolated hASM tissue or homogenates, or to investigate the effects of exposing cultured hASM cells to the inflammatory environment of the asthmatic lung. This could be achieved by treating cultured hASM cells from healthy donors with broncho-alveolar lavage fluid or sputum from control and asthmatic donors.
6.1. Summary of key findings

One aim for this project was to determine if there are transcriptional changes associated with asthma, which are retained in the practised culture conditions of hASM cells from clinically characterised control and asthmatic donors, particularly in relation to airways inflammation, LPA signalling and LPA metabolism. After an initial high-throughput screen of a broad range of potential targets, 12 targets of interest were identified for further, more quantitative study. Of these, two were found to be significantly altered with disease state of the donor; *PDE4B* was down-regulated in severe and moderate asthmatics, while *PPAP2C* was down-regulated in severe asthmatics compared to control. These genes encode a phosphodiesterase isoenzyme and a lipid phosphate phosphatase that can breakdown LPA.

Another aim was to better characterise the actions of a variety of LPA species and other lipids with reported agonist or antagonist activity at a range of LPA receptors. Optimal conditions for a $[^{35}\text{S}]$GTP$_\gamma$S binding assay were developed for assessment of LPA receptor-activation in membranes prepared from RH7777 cells exogenously expressing human LPA$_1$. Though no LPA response was observed in membranes derived from wild-type cells, expression of LPA$_1$, without any agonist stimulation, was enough to stimulate a high level $[^{35}\text{S}]$GTP$_\gamma$S binding, which may indicate constitutive activity of LPA$_1$ or a significant concentration of LPA endogenous to the membranes generated. All $[^{35}\text{S}]$GTP$_\gamma$S binding, including the basal component, was ablated by pre-treatment of the membranes with PTx, indicating that the response observed in LPA$_1$ membranes is $G\alpha_i$-mediated and that the high basal level is receptor-mediated.

This assay was then successfully applied to determine the effects and potency of a range of LPA species and other lipids that are reportedly active at LPA receptors. LPAs with fatty acid chains of varying length and saturation, linked by acyl or alkyl groups at either the sn-1 or sn-2 positions were all active at LPA$_1$. There was no particular pattern for LPA potency, though LPAs with 18:0 acyl chains were least potent. Assessment of other lipids that are reportedly active at the LPA receptors was largely in agreement with established findings; VPC 31143, VPC 31144 and (2S)-OMPT were found to have agonist activity, while DDP, VPC 32179, VPC 32183 and VPC 12249 showed antagonist activity. Though some studies have shown effects of
N-palmitoyl-L-serine-PA and N-palmitoyl-L-tyrosine-PA on signalling events mediated through endogenously expressed receptors in *Xenopus* and human cells (Bittman *et al.*, 1996; Liliom *et al.*, 1996; Hooks *et al.*, 1998), these compounds showed no activity at hLPA₁ in this study.

It was intended that this work be continued using membranes isolated from cell lines expressing other LPA receptor subtypes so that any subtype-specific effects of the LPAs and lipids tested could be identified. After failed attempts to generate further LPA receptor-expressing cell lines using the RH7777 background, this work was continued using a Chem-1 cell background over-expressing Ga₁₅ to facilitate coupling to a Ca²⁺ read out. Unexpectedly, wild-type Chem-1 cells displayed robust LPA responses of an indistinguishable magnitude and potency to their LPA receptor-transfected counterparts. This precluded use of the Chem-1/Ga₁₅ cells for investigation of LPA receptor subtype-specific effects. Observed LPA and lipid responses were similar to those seen for [³⁵S]GTPγS binding in RH7777-LPA₁ cell membranes, though the range of potencies observed in each cell-line was narrower, perhaps due to the signal amplification that comes with measuring a downstream event such as Ca²⁺ mobilisation.

Since LPA responses were observed in a model cell-line thought to be LPA receptor-null, RT-PCR analyses of many of the cell types used in this project were performed. Multiple LPA receptor mRNAs could be detected in each cell type tested. All rat cell-lines tested endogenously expressed *rLpar2*, *rLpar5* and *rLpar6*, while all human cell-types tested endogenously expressed *hLPAR1*, *hLPAR2*, *hLPA3* and *hLPAR6*.

In the final Results Chapter, I aimed to characterise LPA-mediated signalling events and investigate any disease-dependent trends in cultured primary hASM cells. Work focussed on two signalling read-outs of potential significance to asthma pathophysiology which are known to be altered by LPA treatment; cAMP and cell migration. LPA did not induce a cAMP response in hASM cells when added alone but was able to modulate cAMP responses stimulated by either forskolin (FK) or isoprenaline as agonists. Direct AC-stimulatory or β₂-adrenoceptor-mediated cAMP responses were higher in cells from asthmatic donors compared to controls, though the reasons for this are unclear. LPA inhibited FK-induced cAMP responses in a concentration-dependent manner, but induced a biphasic effect on β₂-adrenoceptor-mediated cAMP responses. The effects of treatment with 1 μM LPA on AC- and β₂-
adrenoceptor-mediated cAMP responses were completely prevented by pre-treatment with the LPA1/3-selective antagonist Ki16425. Though LPA was shown to modulate cAMP responses in hASM cells, no disease state-dependent effects of LPA were apparent.

Though hASM cells were the primary focus of this work, SW982 cells were also used as a model of LPA1-dependent signalling in human cells to facilitate assay-development and further investigations. Concentration-dependent inhibition of FK responses and biphasic effects of LPA treatment on β2-adrenoceptor-mediated cAMP responses were recapitulated in this model cell line. Further investigation revealed that the inhibitory phase of LPA action on β2-adrenoceptor-mediated cAMP response is Gαi-mediated.

In SW982 cells, LPA potently induced time- and concentration-dependent non-directional cell migration. The effect was substantially greater than that induced by serum and was completely inhibited by pre-treatment with PTx or the LPA1/3-selective antagonist, Ki16425. LPA-induced SW982 cell migration was also significantly attenuated by treatment with inhibitors of ROCK, MAPKK and PI3K. Despite extensive efforts to apply this Transwell method to hASM cells, no hASM cell migration could be observed.

Figure 6.1 provides a summary of the findings in this work.
A  Control

B  Asthma
Summary of the work presented in this thesis. In hASM, LPA modulates cAMP production, whether mediated by β2-adrenoceptors (β2AR) or by direct activation of adenylyl cyclase (AC; Figure 5.5). Though several LPA receptor subtypes are expressed in hASM, at least at the RNA level (Table 4.1), a functional role has been demonstrated for LPA₁ or LPA₃ receptor subtypes only (Figure 5.6). No disease state-dependent differences in LPA receptor expression or function have been detected in hASM (Figure 3.6, Figure 3.7 and Figure 5.5). Work in the SW982 model cell line indicates that cAMP modulation is mediated, in part, by Gαᵢ inhibition of AC (Figure 5.11), while work in the RH7777 model cell line indicates that several different LPA species may contribute to LPA receptor activity (Figure 4.7). PDEs are active in hASM (Figure 5.2) and PDE4B expression was found to be down-regulated at the RNA level in hASM cells from asthmatics (Figure 3.10). The current literature indicates increased LPA levels in the asthmatic airway (see Introduction), and the observed downregulation of LPP2-encoding RNA in hASM (Figure 3.10) may contribute to this. The overall effect of these combined factors on cAMP production in the physiological setting is unknown.
6.2. Discussion

The expression analysis performed here showed that \textit{PDE4B} mRNA was down-regulated in the severe and moderate asthmatics investigated. Expression studies of phosphodiesterase (PDE) isoenzymes are challenging, since there are so many variants. There are 11 PDE families, each containing multiple subtypes, such that there are over 20 genes encoding over 50 PDE proteins in mammals (Keravis and Lugnier, 2012). Despite the interest in PDE function in airways disease (Page and Spina, 2012), expression analysis of PDE isoenzymes has not yet been performed in hASM cells derived from asthmatics, presumably due to the inevitable technical difficulties that arise from investigating such a large family. \textit{PDE4B} expression has not previously been assessed in hASM cells from control and asthmatic subjects. Efforts in airways have focussed on PDE4D as this isoenzyme is thought to be the functionally dominant variant (see previous discussion), but the findings presented here prompt further examination. Further investigation of the effect of asthma on PDE expression in hASM cells would be of particular interest due to the unexpected increase in cAMP accumulation observed in cells from asthmatic donors. Since increased cAMP production is observed in hASM cells from asthmatics when stimulating with FK or isoprenaline, this effect must originate from a convergent signalling component, e.g. AC activity, PDE activity or G protein activity. It is tempting to speculate that since I have observed a down-regulation of \textit{PDE4B} mRNA expression in severe asthmatics, the increased cAMP output from hASM cells from asthmatic donors is caused by PDE dysfunction. However, all experiments carried out here used the pan-PDE inhibitor, IBMX, so cannot shed any light on the involvement of PDE activity in this disease state-dependent effect. Still, it is also worth noting that an adenylyl cyclase isoenzyme, AC8, and the gene encoding Gαs were identified in the initial screen for genes of interest for the expression analysis presented in Chapter 3. Unfortunately, there was not sufficient transcript present in the samples obtained here for accurate quantitative expression analysis of these targets. It is possible that there is dysfunction at a fundamental level of cAMP production regulation in asthmatics and this warrants further investigation.

While I have shown that \textit{PPAP2C} was down-regulated in severe asthmatics, little is known about its protein product, LPP2. Follow-up studies should begin by ensuring that
this down-regulation at the transcriptional level is reflected in protein expression. Investigations into LPP2 expression in airway tissues, and any respiratory disease-dependent effects would be of particular interest, though it should be noted that the investigations of LPA-induced signalling in control- and asthmatic-derived hASM cells performed here do not suggest any specific disease-dependent changes in LPA responses. Investigation of the half-life of $^{32}$P-labelled LPA in wild-type and $Ppap2a$ (LPP1) knockout mice has shown that LPP1 regulates LPA levels in vivo (Tomsig et al., 2009). Although $Ppap2c$ knockout animals have been generated and shown to be grossly normal (Zhang et al., 2000), no in vivo investigations of the physiological function of LPP2 have been performed. It would be interesting to assess the impact of LPP2 knockout on circulating LPA levels using similar methods to Tomsig et al. (2009). If LPP2 is found to regulate LPA levels, the effects of LPP2 knockout on LPA in the airway, e.g. in BAL fluid or sputum, should be investigated, since a potential role for LPP2 dysfunction in asthma has been identified here. Since LPA levels are thought to be increased in the asthmatic airway (Georas et al., 2007), LPP2 expression could also be assessed in murine models of asthma.

Since completing this work, the crystal structure of autotaxin (ATX) has been solved (Hausmann et al., 2011; Nishimasu et al., 2011). Analysis of the binding pocket shows that it has optimal dimensions to accommodate a 14:0 fatty acyl group, but is able to accommodate acyl chains of varying length in different conformations; for example, by allowing saturated chains to adopt a straight conformation and unsaturated chains to bend at their double bonds (Nishimasu et al., 2011). I, and others, have shown that LPA$_1$ is activated by LPA with a range of acyl chain lengths, so it is possible that the LPA binding site of the LPA receptors is similarly arranged to allow the observed flexibility in ligand-binding. I found that LPAs with an 18:0 acyl chain were the least potent at LPA$_1$ and this may be due to inefficient ligand-binding caused by the long chain length coupled with the inflexibility of complete saturation. Autotaxin is now thought to interact directly with LPA receptors (Tabchy et al., 2011) and there is the potential for ATX function to profoundly affect LPA receptor function. It seems that better understanding of ATX-LPA receptor interactions will be a major focus for the field in the coming years and will aid understanding of LPA receptor function.
When investigating the pharmacological profile of LPA receptors, I encountered an obstacle that affects research into any receptor with such a wide expression pattern. Expression of some LPA receptor subtypes is near-ubiquitous and it is likely that many cell types simultaneously express multiple LPA receptor subtypes, as seen with all cell-lines tested here. This presents some difficulties when trying to attribute the actions of LPA to any one LPA receptor-subtype. This is confounded by the fact that there are few LPA receptor subtype-selective antagonists available and no well-characterised antibodies that can distinguish expression of the LPA receptor subtypes. This might be particularly impeding for drug discovery where investigation of receptor subtype-specific effects are critical. One aim of this project was to better characterise the activity of several LPA species and other lipids at the LPA receptors. This was hampered by the fact that the Chem-1 cells used here expressed endogenous LPA receptor mRNAs and exhibited potent responses to LPA stimulation. Though they have been shown to express LPA receptor mRNA (Lee et al., 2006), the non-transfected RH7777 cell membranes used in the initial stages of this project exhibited no LPA-induced $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding, so this work could be continued using this cell background instead of the Chem-1 cells. This will require a significant investment of time to generate the required LPA receptor-expressing cell-lines, but would provide some much needed clarification on the effects of the available lipid compounds that reportedly affect LPA receptor activity.

6.2.1. **What can be learnt about the role of LPA in respiratory disease?**

The over-reaching aim for this project was to investigate the potential role that LPA, acting through its receptors, has in airways disease, using cultured cells from clinically characterised donors with and without clinically characterised asthma. I have shown that several LPA receptor subtypes are expressed by the human cell types investigated here and that LPA does indeed regulate cAMP signalling in hASM cells, but I have not observed any disease state-dependent effects of LPA. Though LPA regulation of cAMP has previously been studied in hASM cells (Ediger and Toews, 2001), this has not previously been studied in health and disease, so these are novel data.

Perhaps it is unsurprising that LPA receptor activity *per se* is unchanged in disease, though there is evidence to suggest that the amount of LPA present in the asthmatic lung is increased (Georas et al., 2007). The expression analysis performed here showed
that an enzyme capable of breaking down LPA, LPP2, is down-regulated in severe asthmatics. Though much more work is needed to confirm this result in a physiological setting, this is one potential causal route for the increased LPA levels observed in asthmatics. The cellular and molecular effects of increased LPA in the airways remain to be determined, though it is likely that effects on cAMP production, like those observed here, would contribute. Since cAMP is critical in regulating contractility and inflammation in hASM cells, LPA remains a target of interest for inflammatory airways disease.


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