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

Thesis Submitted for the Degree of Doctor of Philosophy at the University of Leicester

Marie Valente

Department of Cell Physiology and Pharmacology

University of Leicester

March 2014

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}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.

I have had great lab-mates and fellow PhD students during my studies and I am very grateful for their help, friendship and particularly for helping me keep perspective during the rough patches. Special thanks go to my lab-mates Sophie and Roopdog for making the lab a more entertaining place to work, in spite of Heart FM, and to lab-veteran Raj for his technical advice, but mostly for his mood-lifting karaoke. I am grateful to the many fellow researchers that have taught me various methods and patiently tried to answer all my questions; to Danijela and Edith for molecular biology methods, to Gav and Carl for help with the microscope and migration assay, to Raj for his expertise on signalling assays, to Gary for advice on stats (and bikes) and to Sophie for offering help and advice on just about everything else. I am also very grateful to the technicians and researchers at Glenfield hospital involved in establishment of human cell-cultures.

I would also like to thank the lab at Novartis for kindly welcoming me into the fold and making me part of their social group while I was visiting - the murder mystery is particularly memorable. Special thanks go to Afrah, Liz and Sarah for their patient help with various methods and machinery and to Mark, Toby and Stephen for their scientific advice and guidance. I am also very grateful to the Kent and Bradley households for welcoming me into their families and giving me a place to stay while in Horsham.

Lastly, I owe a huge thank you to my family and friends outside the lab. They have all listened patiently while I've moaned about my PhD and have suffered my negligence with understanding in these last few months. Thank you to Mum, Dad, John, Andrew and Frances for their support and unwavering belief in me and to Chad, without whose patience, encouragement and perseverance, I would not have got this far.

Contents

Abbreviations

AC	Adenylyl cyclase
AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
AP-1	Activator protein 1
AR	Adrenoceptor
ASM	Airway smooth muscle
ATP	Adenosine 5'-triphosphate
ATX	Autotaxin
BAL	Bronchoalveolar lavage
bp	Base pairs
BSA	Bovine serum albumin
c.p.m.	Counts per minute
CaM	Calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Adenosine 3',5'-cyclic monophosphate
cDNA	Complementary DNA
CDS	Coding DNA sequence
COPD	Chronic obstructive pulmonary disorder
CRE	cAMP response element
CREB	cAMP response element-binding protein
Ct	Cycle threshold
DAG	Diacylglycerol
DDP	Dodecyl fatty acid phosphate
DGPP	Diacylglycerol pyrophosphate
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPM	Disintegrations per minute
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
Edg	Endothelial differentiation gene
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethyleneglycoltetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAF-BSA	Fatty acid-free bovine serum albumin
FBS	Fetal bovine serum
FDSS	Functional drug screening system
FK	Forskolin
FPP	Farnesyl pyrophosphate
Fura-2-AM	Fura-2-acetoxymethylester
G protein	Guanine nucleotide-binding protein
G418	Geneticin sulphate
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
GDP	Guanosine 5’-diphosphate
GEF	Guanine nucleotide exchange factor
GINA	Global initiative for asthma
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	Guanosine 5’-triphosphate
GTPγS	Guanosine 5'-O-[γ -thio]triphosphate
hASM	Human airway smooth muscle
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IBMX	3-isobutyl-1-methylxanthine
IFN	Interferon
IL	Interleukin
IP_3	Inositol 1,4,5-trisphosphate
IPF	Idiopathic pulmonary fibrosis
Iso	Isoprenaline
JNK	c-Jun N-terminal kinase
KHB	Krebs-Henseleit buffer
LCAT	Lecithin-cholesterol acyltransferase

LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPP	Lipid phosphate phosphatase
LPS	Lysophosphatidylserine
LysoPLD	Lysophospholipase D
MAPK	Mitogen-activated protein kinase
MIF	Macrophage migration inhibitory factor
NAG	*N*-Arachidonylglycine
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
NSB	Non-specific binding
PA	Phosphatidic acid
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	Ca^{2+}-dependent protein kinase
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PMSF	Phenylmethanesulfonylfluoride
PS	Phosphatidylserine
PTx	Pertussis toxin
PVDF	Polyvinylidene fluoride
RGS	Regulator of G protein signalling
ROCK	Rho-dependent kinase
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
S1P	Sphingosine-1-phosphate

SDS	Sodium dodecyl sulphate
SOCS1	Suppressor of cytokine signalling 1
SPA	Scintillation proximity assay
SRE	Serum response element
ST2	Suppression of tumorigenicity 2
TBS-T	Tris-buffered saline-Tween
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TLDA	TaqMan® low density array
Tm	Melting temperature
TNFα	Tumour necrosis factor α
Tris	Tris(hydroxymethyl)aminomethane
UDG	Uracil-DNA glycosylase
UV	Ultraviolet
Y-27632	*Trans*-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride

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 1.1**A**). 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

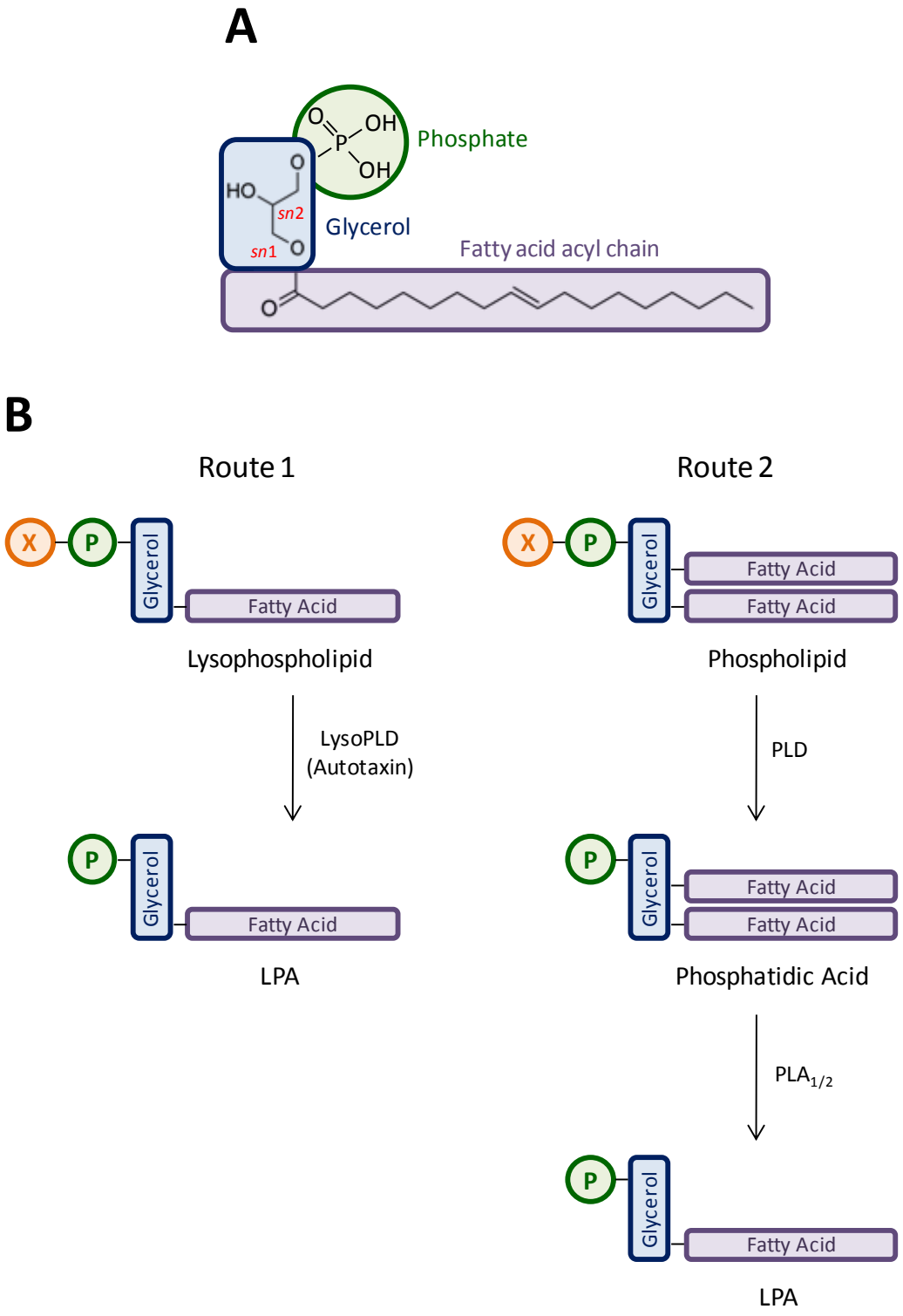


Figure 1.1

A, structure of LPA, highlighting its phosphate, glycerol and fatty acid acyl groups and illustrating the position of the *sn*1 and *sn*2 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 1.1**B**, Route 1) or, alternatively, phospholipids can be converted by a phospholipase D (PLD) to phosphatidic acid, which is then deacylated by a phospholipase A_1 or A_2 ($PLA_{1/2}$) to form LPA (Figure 1.1**B**, 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 PLA_1 isoenzymes, mPA-$PLA_{1\alpha}$ and mPA-$PLA_{1\beta}$, 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-$PLA_{1\alpha/\beta}$ have been found to localise to detergent-resistant portions of the plasma membrane (Hiramatsu *et al.*, 2003). Interestingly, mutations in the mPA-$PLA_{1\alpha}$ isoenzyme are linked to similar genetic hair-loss diseases (Kazantseva *et al.*, 2006; Ali *et al.*, 2007) to those linked with mutation of LPA_6 (Pasternack *et al.*, 2008). These studies support the idea that mPA-$PLA_{1\alpha}$ 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 1.2**A**).

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

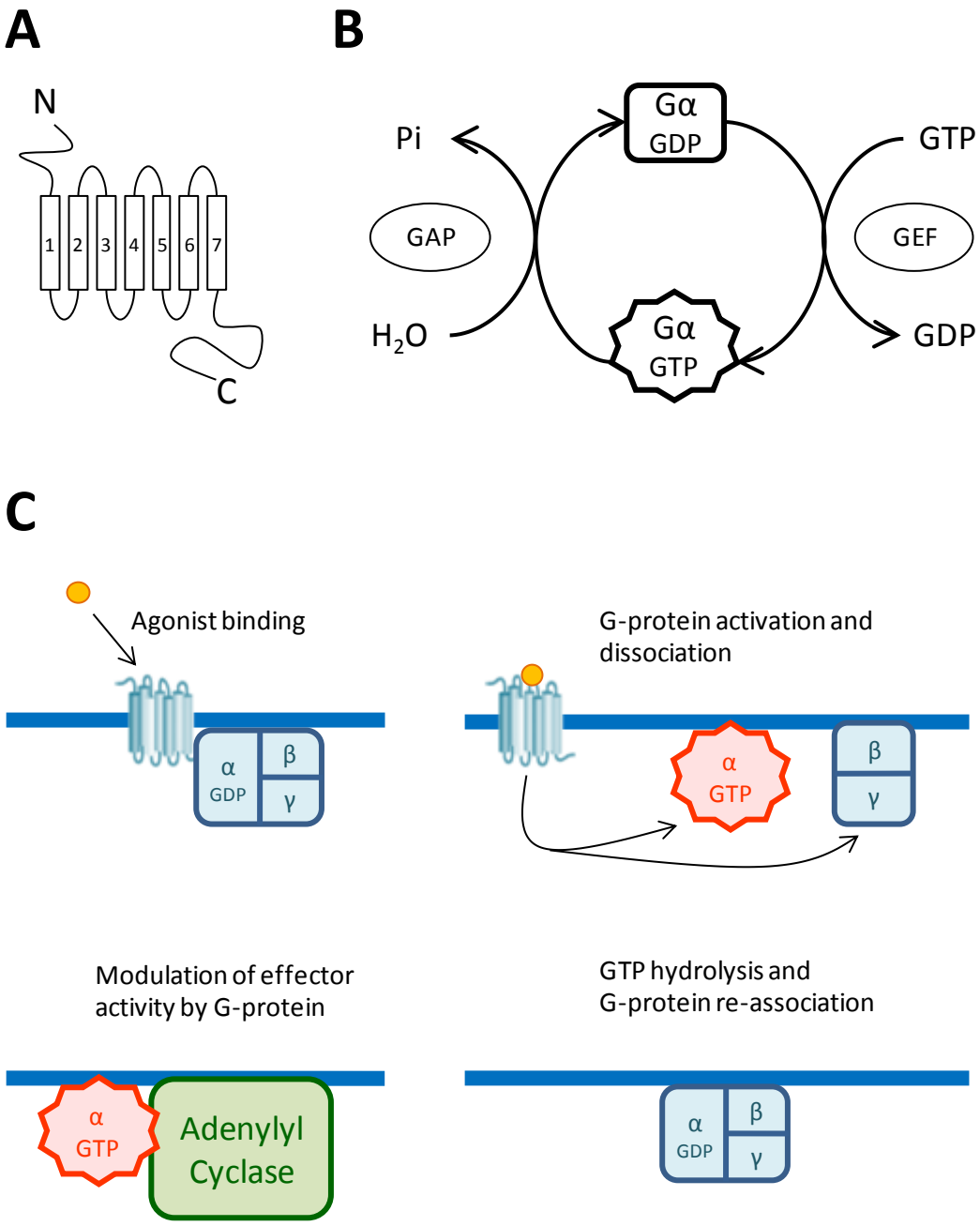


Figure 1.2

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.

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 α, β and γ subunits. The β and γ subunits are closely associated through a tight, coiled-coil interaction and both Gα and Gβγ 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 1.2**B)**. In its activated state, Gα

is bound to GTP and dissociates from Gβγ; the GTP is then hydrolysed by the intrinsic GTPase activity of the Gα subunit to form the inactive, GDP-bound Gα which then re-associates with Gβγ, as shown in Figure 1.2**C** (Bourne *et al.*, 1990).

The intrinsic GTPase activity of Gα can be facilitated by GTPase-activating proteins (GAPs), which enhance the often slow intrinsic catalytic activity of Gα. A large family of proteins possessing RGS (regulator of G protein signalling) domains have been characterised and are considered the major modifiers of Gα 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 Gα-GTP from Gβγ. Both the Gα-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 Gα 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 Gα-subunit that dictates the coupling of the receptor to its downstream effectors. There are four families of Gα, which are grouped according to structural similarity: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Strathmann and Simon, 1991). $G\alpha_s$ 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). $G\alpha_{i/o}$ was identified shortly afterwards and was found to inhibit AC and thus reduce $[cAMP]_i$ (Bokoch *et al.*, 1984). $[cAMP]_i$ 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 $[cAMP]_i$ 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 $[cAMP]_i$ conditions the regulatory subunits are bound to the catalytic subunits and sterically inhibit their activity. When $[cAMP]_i$ 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 $G\alpha_{q/11}$ family can regulate the activity of another membrane-associated enzyme, phospholipase C (PLC). PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2), producing diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) which can both function as signalling molecules. While DAG stays within the plasma membrane, IP_3 is water-soluble and diffuses into the cytoplasm, allowing it to reach its effector, the IP_3 receptor (IP_3R) – a Ca^{2+} channel associated with the endoplasmic reticulum (ER) membrane. Similarly, the DAG released can also go on to effect activation of the Ca^{2+}-dependent protein kinase (PKC). IP_3R activation by IP_3 binding causes release of Ca^{2+} from the intracellular stores of the ER, thus greatly increasing the $[Ca^{2+}]_i$. Like cAMP, Ca^{2+} is an important second messenger that can go on to activate myriad downstream effectors, including proteins with a calmodulin domain. Where $[cAMP]_i$ is regulated by synthesis and degradation, $[Ca^{2+}]_i$ is governed by tightly controlled storage and release (Berridge and Irvine, 1989; Lipp and Reither, 2011).

In 1991, a fourth family of Gα subunit, $G\alpha_{12/13}$, was discovered by Strathmann and Simon. Both $G\alpha_{12}$ and $G\alpha_{13}$ proteins are ubiquitously expressed and share ~67 % amino acid sequence identity in mice (Strathmann and Simon, 1991). Many GPCRs couple to $G\alpha_{12/13}$, including the receptors for LPA (Hains *et al.*, 2006), however, all GPCRs thus far shown to couple $G\alpha_{12/13}$, also couple to other heterotrimeric G proteins (Riobo and Manning, 2005; Siehler, 2008). $G\alpha_{12/13}$ proteins can act via G proteins of the Ras superfamily, rather than by altering cellular concentrations of a small molecule mediator as for $G\alpha_s$, $G\alpha_{i/o}$ and $G\alpha_{q/11}$. GTP-bound $G\alpha_{12/13}$ stimulates the activity of a

RhoGEF thereby activating the small GTPase, RhoA. $G\alpha_{12/13}$ 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 $G\alpha_{12/13}$, 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 Ca^{2+} 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 β_2-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 β_2-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 $G\alpha_{i/o}$ and $G\alpha_s$ 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 $G\alpha_{i/o}$ 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 $G\alpha_s$, which inhibits the intrinsic GTPase activity of $G\alpha_s$ 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 $G\alpha_{q/11}$-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 [^{3}H]inositol labelling of cells and subsequent anion-exchange chromatography to recover the inositol phosphate fraction (Skippen *et al.*, 2013). While $G\alpha_{q/11}$-specific antagonists have been developed (Takasaki *et al.*, 2004), these are not well characterised and are not commercially available.

For $G\alpha_{i/o}$ and $G\alpha_s$, changes in $[cAMP]_i$ can be measured by a number of assays, including a [^{3}H]adenine prelabelling assay (Shimizu *et al.*, 1969) and a cAMP/[^{3}H]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, streptavadin-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 $G\alpha_{12/13}$ 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 [^{35}S]GTPγS binding alongside immunoprecipitation of either RhoA or $G\alpha_{12/13}$ (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.*,

2006) 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 $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_s$ 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 $G\alpha_{q/11}$-mediated activation of phospholipase C and Ca^{2+} mobilisation (Ohata *et al.*, 1997; Zhou *et al.*, 1999), $G\alpha_{i/o}$-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), $G\alpha_{12/13}$-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 $G\alpha_s$-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

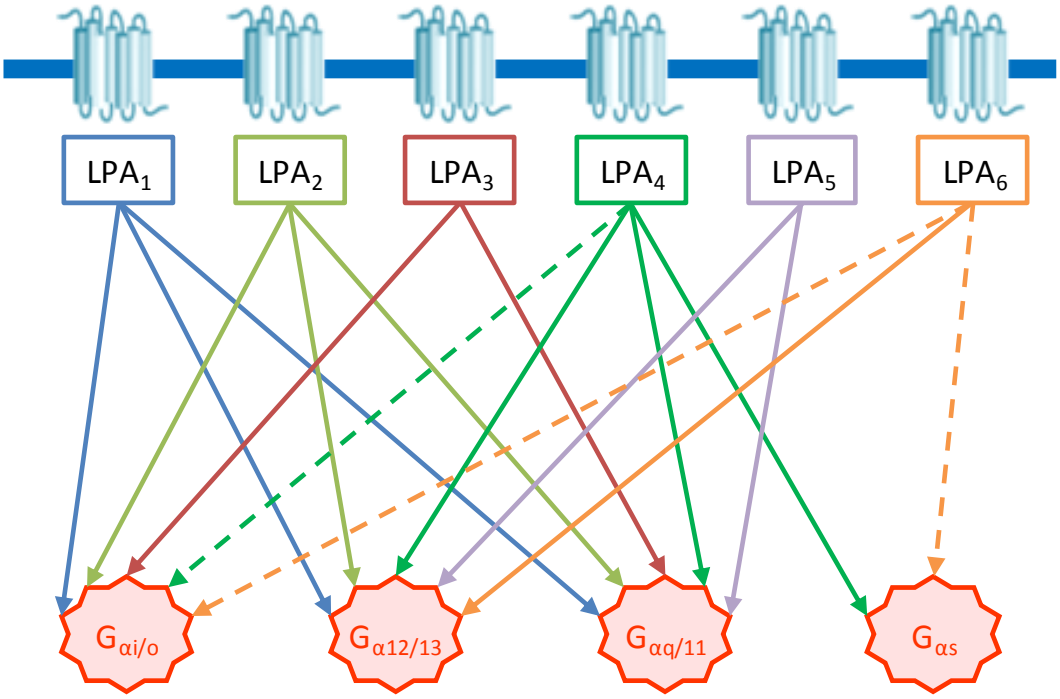


Figure 1.3

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 LPA_1 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 LPA_{1-3} 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, LPA_4, LPA_5 and LPA_6, are structurally more closely related to P2Y purinergic receptors than to the so-called Edg family LPA receptors (LPA_1, LPA_2 and LPA_3) and S1P receptors. LPA_4, LPA_5 and LPA_6 are thought to have evolved from a distinct lineage to the Edg family LPA receptors (Yanagida and Ishii, 2011).

1.3.1.1. *LPA_1*

LPA_1 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). LPA_1 has been shown to couple to multiple G proteins: $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Fukushima *et al.*, 1998; Ishii *et al.*, 2000). Its expression is widespread, but is enriched in the brain during development and LPA_1-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, LPA_1 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).

LPA_1 has been implicated in a broad range of physiological and pathophysiological processes. Roles for LPA_1 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 LPA_1 deletion may serve as a mouse model for schizophrenia, or other psychiatric disease (Harrison *et al.*, 2003; Roberts *et al.*, 2005). LPA_1 has been implicated in the myelination process in both the peripheral and central nervous system and there is thought to be LPA_1 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 LPA_1 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, LPA_1 is being pursued as a therapeutic target by several fields, though it should be noted that effects of LPA_1 activation are not consistent across all cell types. For example, there are some reports of LPA_1 mediating protection from tumourigenesis (Kato *et al.*, 2012).

1.3.1.2. *LPA_2*

LPA_2 was identified by its sequence similarity to LPA_1 (Contos and Chun, 2000) and has been shown to couple to $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ proteins (Ishii *et al.*, 2000). The expression pattern of LPA_2 is somewhat more restricted than LPA_1, 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 LPA_2 might have a role in supporting normal LPA_1 function (Contos *et al.*, 2002). Demonstrated functions of LPA_2 activation include cell migration and survival, and LPA_2 has been most intensively studied in cancer, particularly gynaecological cancers (Zheng *et al.*, 2001; Deng *et al.*, 2002). LPA_2 is up-regulated in ovarian cancer cells and LPA_2 expression has been linked to invasiveness of ovarian cancer cell tumours in a xenograft mouse model (Yu *et al.*, 2008; Goetzl *et al.*, 1999). LPA_2 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 $G\alpha_{12/13}$/Rho-dependent mechanism in pancreatic cancer cells (Komachi *et al.*, 2009).

1.3.1.3. *LPA_3*

LPA_3 couples to $G\alpha_{q/11}$ and $G\alpha_{i/o}$, but coupling to $G\alpha_{12/13}$ 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-$PLA_{1\alpha/\beta}$ (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 $G\alpha_{12/13}$-mediated neurite retraction and cell rounding in LPA_4-expressing B103 cells, using either $G\alpha_{12}$ and $G\alpha_{13}$ 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 $G\alpha_s$ 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 $G\alpha_{q/11}$- and $G\alpha_{i/o}$-mediated component, whereas Yanagida *et al.* (2007) reported that this response could be completely blocked by the $G\alpha_{q/11}$ 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 [^{3}H]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 pyrosphosphate has also been identified as a ligand for LPA_3 and can inhibit LPA-induced Ca^{2+} mobilisation in RH7777 cells over-expressing LPA_3 (Liliom *et al.*, 2006). While LPA might be the dominant ligand for GPR92/LPA_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_5 by the International Union of Basic and Clinical Pharmacology (Davenport *et al.*, 2013). LPA_5 couples to $G\alpha_{q/11}$ and $G\alpha_{12/13}$ protein families (Lee *et al.*, 2006).

LPA_5 shows preference for alkyl-linked LPA species (Williams *et al.*, 2009), while LPA_1, LPA_2 and LPA_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_5 is the platelet-type LPA receptor since *LPAR5* mRNA is highly expressed in platelets, and RNAi knockdown of LPA_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_1 and LPA_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_5 may mediate the chemorepellent roles of LPA. LPA_5 was shown to mediate LPA- and autotaxin-induced chemorepulsion in certain cancer cell-lines using siRNA receptor knockdown. As observed for LPA_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_6*

The orphan receptor 6H1 was initially identified as a purinoceptor and termed $P2Y_5$ (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). LPA_6 is thought to couple to the $G\alpha_{12/13}$ protein family (Yanagida *et al.*, 2009).

Its role in hair development is the best characterised function of LPA_6 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-PLA_1, 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 LPA_3, 2-acyl LPA species, like those produced by mPA-PLA_1, are more potent at LPA_6 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 $LPA_{4\text{-}6}$ receptors, it has also been suggested that GPR87, GPR35 and $P2Y_{10}$ 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 LPA_3 and LPA_6, 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). LPA_1 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); LPA_2 knockouts have no obvious phenotypic changes (Contos *et al.*, 2002) and female LPA_3 knockouts have altered embryo spacing and delayed implantation (Hama *et al.*, 2007). $LPA_{1/2/3}$ 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 $LPA_{1/2/3}$ triple knockout, a substantial proportion of *in vivo* LPA function must be attributable to other entities such as $LPA_{4/5/6}$, 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). Omission of a string of

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 β_3-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, IFNγ, 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 LPA_1 and LPA_3, but not LPA_2 (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_H2 responses, which are more pertinent to atopic asthma. IL-13 is a key mediator of T_H2 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_1 and LPA_2 differentially mediating responses (Goetzl *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^{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 (Cerutis *et al.*, 1997; Zhao *et al.*, 2011). Furthermore, studies show that LPA can accelerate wound healing *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 $G\alpha_{i/o}$ and $G\alpha_{q/11}$ involvement, while in lung epithelial cells PKCδ 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 adenylyl 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 $G\alpha_{i/o}$ 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 LPA_2 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 LPA_2 knockout significantly attenuated levels of T_H2 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, geneticin sulphate (G418), native *Taq* DNA polymerase, Express SYBR® GreenER™ qPCR SuperMix and Fluo-4-AM (fluo-4-acetoxymethylester) were from Invitrogen, California, USA. [^{3}H]cAMP and [^{35}S]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 1 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 LPA_1 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.

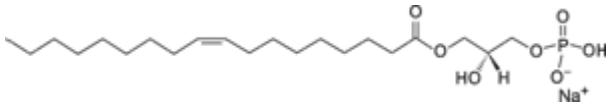
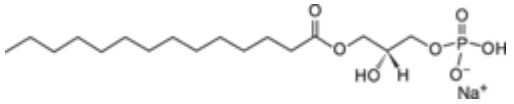
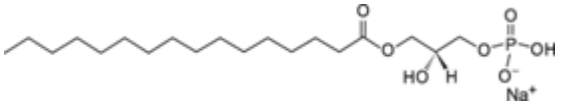
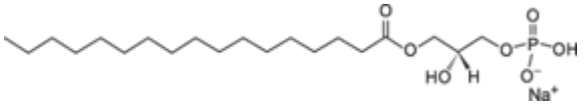
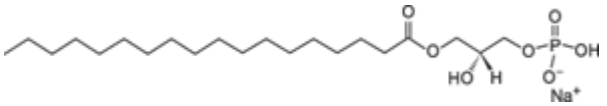
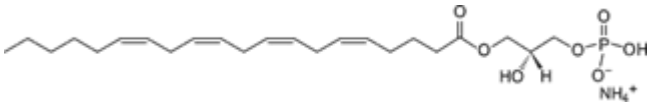
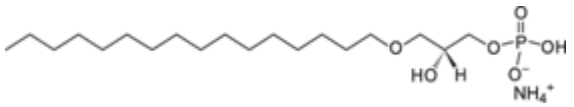
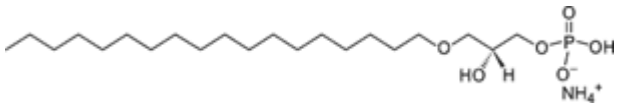
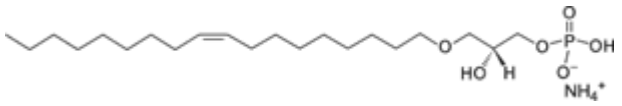
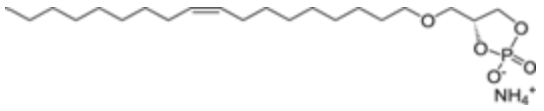
LPA	Chemical Structure
18:1	
14:0	
16:0	
17:0	
18:0	
20:4	
C16:0	
C18:0	
C18:1	
Cyclic C18:1	

Table 2.1

Table showing chemical structures of LPA species used in the pharmacological profiling study.

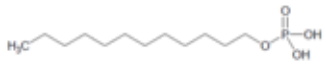
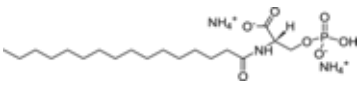
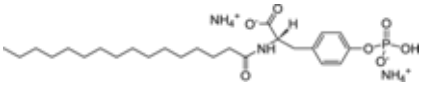
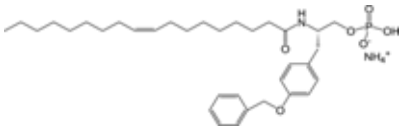
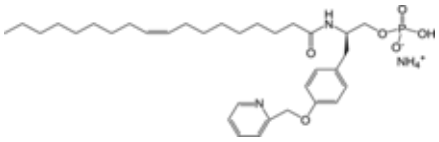
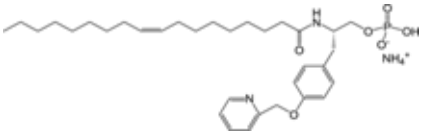
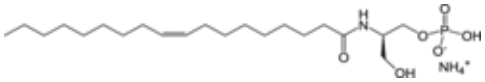
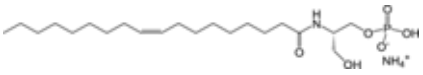
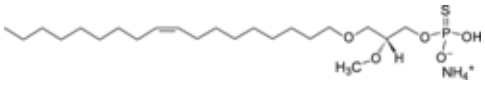
Lipid	Chemical Structure	Reported Activity
DDP		Reported competitive inhibitor at LPA_1 and LPA_3 and agonist at LPA_2
N-P-Serine-PA		Some controversy
N-P-Tyrosine-PA		Some controversy
VPC12249		Reported LPA_1 and LPA_3 selective antagonist
VPC 32179 (*R*)		Reported LPA_1 and LPA_3 selective antagonist
VPC 32183 (*S*)		Reported LPA_1 and LPA_3 selective antagonist
VPC 31143 (*R*)		Reported LPA receptor agonist
VPC 31144 (*S*)		Reported LPA receptor agonist
2(*S*)-OMPT		Reported LPA_3 selective agonist

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 LPA_1 or LPA_3 were purchased from Millipore. Chem-1 cell lines stably transfected to express LPA_2 were developed by Novartis.

All cell types were maintained by incubation at 37 °C in a 5 % CO_2-air humidified atmosphere. When confluent, flasks of cells were passaged by washing twice with PBS (without Ca^{2+}/Mg^{2+}) 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 % CO_2-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 cm^2 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 RNAse 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:

Gene Name	NCBI Accession Number	Forward Primer			Reverse Primer			Product length
		Start residue	Sequence	T_m	Start residue	Sequence	T_m	
RN18S1	NR_003286	891	GTTGGTTTTCGGAACTGAGG	57.3	1090	GCATCGTTTATGGTCGGAAC	57.3	200
h*LPAR1*	NM_001401.3	834	GCGGGTAGTGGTGGTCATTGTG	64.0	1403	ATGGTGTGGTTGAGGGAGGAAGC	64.2	570
r*Lpar1*	NM_053936.3	316	CTGGGCATCACTGTCTGCGTGTTC	66.1	963	GCAGACAATAAAGGCACCCAGCAC	64.4	648
h*LPAR2*	NM_004720.5	157	GTGCTACTACAACGAGACCATCGG	64.4	442	AAGTGAAAGTCGGGCTGTGCG	61.8	286
r*Lpar2*	NM_001109109.1	393	CTCTCCATCAAAGGCTGGTTCCTG	64.4	567	CCACCCACACTCCCACGATGA	63.7	175
h*LPAR3*	NM_012152.2	436	GCTGCCGATTTCTTCGCTGGA	61.8	575	AAGTTGGTGAGGGAAGCAGTCAAG	62.7	140
r*Lpar3*	NM_023969.1	84	TGTGCGTTGGGACATTCTTCTGCC	64.4	218	CAAAGAAATCCGCCACCGCCAAG	64.2	135
h*LPAR4*	NM_005296.2	870	TGCCATTGTGTGTGCTGGTGT	59.8	1229	ACCAGGGCATACAAGAAGAGGACA	62.7	360
r*Lpar4*	NM_001106940.1	723	AGTGCTTCCCTGTTTGTCTTCTGC	62.7	1112	AGTGGTGGTCGCATTGTTGACG	62.1	390
h*LPAR5*	NM_001142961.1	665	TGCCCGTTCGTCTCTCCTACTACG	66.1	902	ACGGCAAACACCAGGATGAGCG	64.0	238
r*Lpar5*	XM_001063300.2	776	TTCTGCTGTGCTTCGTGCCCT	61.8	1053	TTCTGAGGGTGGTTCGGTGAGC	64.0	278
h*LPAR6*	NM_005767.5	1750	TCATCTGCGTCCTCAAAGTCCGA	62.4	2098	TGGGTAGACTGAACAAAAACGGCG	62.7	349
r*Lpar6*	NM_001045843.1	623	ACGGCTCCCACTGCCCTTATG	63.7	1056	CGCACTTCCTCCCATCACGGT	63.7	434

Table 2.3

Table of sequences and details of primers used for RT-PCR

Component	Final concentration	µL in 50 µL reaction
10 x PCR buffer	1 x	5
50 mM $MgCl_2$	1.5 mM	1.5
10 µM each dNTP mix	0.2 µM	1
10 µM each primer mix	0.2 µM	1
5 U μL^{-1} *Taq* polymerase	1 U per reaction	0.2
DNA template	50 ng cDNA or 1 ng plasmid	Variable
PCR grade water	To volume	To 50 µL

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 $MgCl_2$ were provided by manufacturer with the polymerase.

94 °C	3 min	Initial denaturation of template	
94 °C	45 s	Denaturation	x 30 cycles
x °C	30 s	Annealing	
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_m. Please see Table 2.3 for primer T_m 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, $MgCl_2$, 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:

50 °C	2 min	UDG incubation
95 °C	5 min	Initial template denaturation and enzyme hot-start

95 °C	15 s	Denaturation	x 36 cycles
59 °C	30 s	Annealing	
72 °C	30 s	Extension	
72 °C	10 min	Final extension	

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

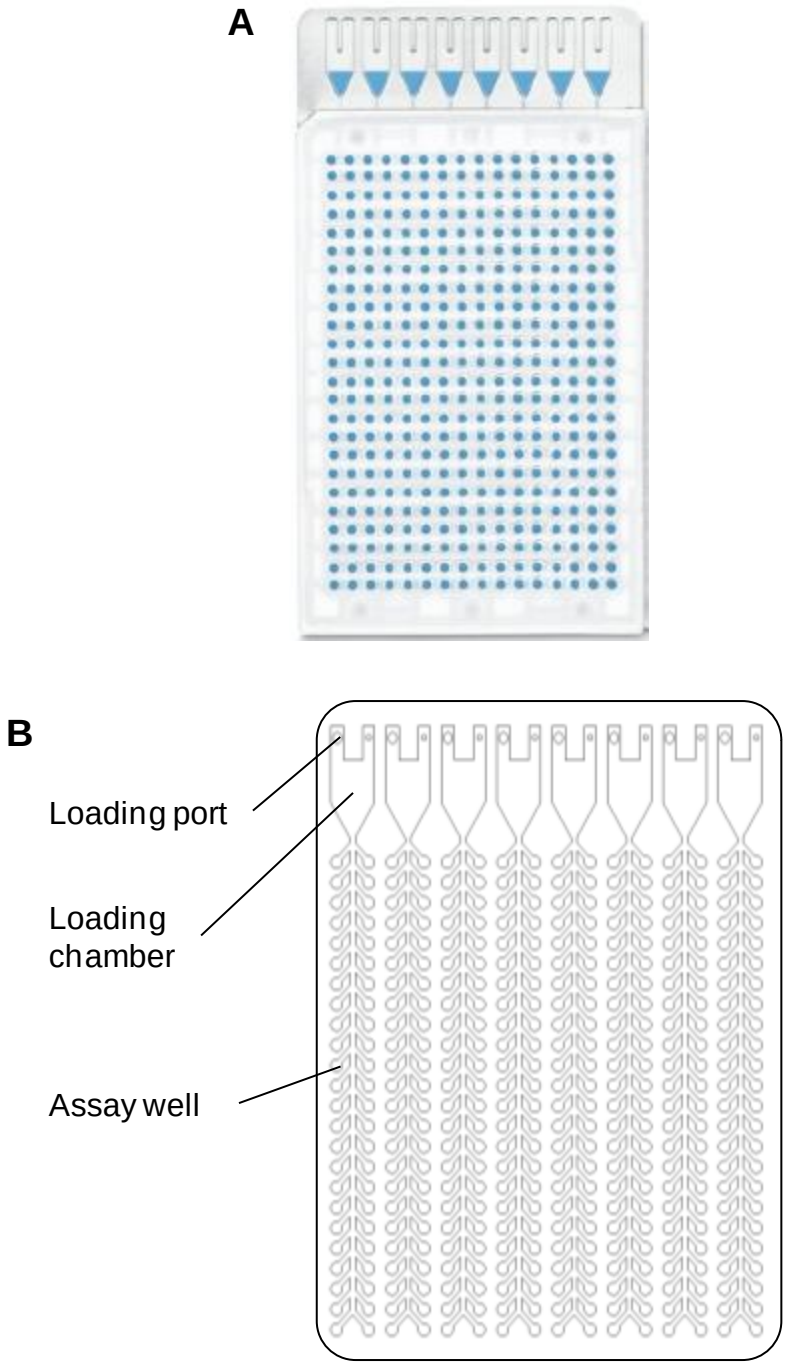


Figure 2.1

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.

IL1A	Interleukin 1α
IL1B	Interleukin 1β
IL4	Interleukin 4
IL5	Interleukin 5
IL6	Interleukin 6
IL8	Interleukin 8
IL9	Interleukin 9
IL10	Interleukin 10
IL13	Interleukin 13
IL18	Interleukin 18
RN18S1	18S rRNA
CCL2	CC ligand 2
CCL3	CC ligand 3
CCL5	CC ligand 5
CCL11	CC ligand 11
CCL19	CC ligand 19
CCL21	CC ligand 21
CSF2	Colony Stimulating Factor 2 (GM-CSF)
SOCS1	Supressor of Cytokine Signalling 1
TNF	Tumour Necrosis Factor
IFNG	Interferon γ
TGFB1	Transforming Growth Factor β1
MMP1	Matrix Metalloproteinase 1
MMP2	Matrix Metalloproteinase 2
MMP7	Matrix Metalloproteinase 7
MMP9	Matrix Metalloproteinase 9
TIMP1	TIMP Metallopeptidase inhibitor 1
TIMP2	TIMP Metallopeptidase inhibitor 2
ACTB	B-Actin
VIM	Vimentin
COL1A1	Collagen 1 α1
COL1A2	Collagen 1 α2
COL3A1	Collagen 3 α1
SPP1	Secreted Phosphoprotein 1
FN1	Fibronectin 1
NOS2	Nitric Oxide Synthase 2 (inducible)
NFE2L2	nuclear factor (erythroid-derived 2)-like 2, (NRF2)
HMOX1	Haemoxygenase 1
VCAM1	Vascular Cell Adhesion Molecule 1
ICAM1	Intercellular Adhesion Moleculte 1
CDH1	Cadherin 1
CDH2	Cadherin 2
CDH12	Cadherin 12
SNAI1	Snail Homolog 1
SNAI2	Snail Homolog 2
SERPINE1	Serine Protease Inhibitor 1 / plasminogen activator inhibitor 1
RELA	RelA

NFKB1	NFκB1
NFKB2	NFκB2
WISP1	Wnt1 Inducible Signalling Pathway protein 1
SEMA6C	Sema domain containing 6C
SEMA7A	Sema domain containing 7A
ENPP2	Autotaxin
ERRFI1	ERBB receptor feedback inhibitor 1
CXCL1	CXC chemokine ligand 1
PDGFA	Platelet derived growth factor A
PDGFB	Platelet derived growth factor B
PDGFC	Platelet derived growth factor C
EGF	Epidermal growth factor
BMPR2	bone morphogenetic protein receptor, type II
SERPINB2	plasminogen activator inhibitor 2
PLAUR	plasminogen activator, urokinase receptor
CCL7	CC chemokine ligand 7 (monocyte chemoattractant protein 3)
CX3CL1	CX3C chemokine ligand 1
ADAM19	ADAM metallopeptidase domain 19
GREM1	Gremlin 1
VEGFB	vascular endothelial growth factor B
BMP4	bone morphogenetic protein 4
FGF18	Fibroblast growth factor 18
THBS1	thrombospondin 1
PIM1	pim-1 oncogene
SPHK1	sphingosine kinase 1
CTGF	connective tissue growth factor
MIF	macrophage migration inhibitory factor
FASLG	Fas ligand (TNF superfamily member 6)
EDN1	Endothelin 1
ACE	angiotensin I converting enzyme 1
ACE2	angiotensin I converting enzyme 2
PDE4A	phosphodiesterase 4A, cAMP-specific
PDE4B	phosphodiesterase 4B, cAMP-specific
PDE4D	phosphodiesterase 4C, cAMP-specific
OSM	Oncostatin M
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)
SPINT1	serine peptidase inhibitor, Kunitz type 1
HGF	hepatocyte growth factor
MUC5AC	Mucin 5AC
NRP1	neuropilin 1
NRP2	neuropilin 2
LAMP2	lysosomal-associated membrane protein 2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HPRT1	hypoxanthine phosphoribosyltransferase -1
PGK1	phosphoglycerate kinase 1

Table 2.5

Table showing gene and gene product names of the targets assayed on the inflammation TLDA.

LPAR1	LPA_1 receptor	ADCY4	adenylyl cyclase 4
LPAR2	LPA_2 receptor	ADCY5	adenylyl cyclase 5
LPAR3	LPA_3 receptor	ADCY6	adenylyl cyclase 6
LPAR4	LPA_4 receptor	ADCY7	adenylyl cyclase 7
LPAR5	LPA_5 receptor	ADCY8	adenylyl cyclase 8
LPAR6	LPA_6 receptor	ADCY9	adenylyl cyclase 9
ENPP2	Autotaxin	ACTB	B-actin
LCAT	lecithin-cholesterol acyltransferase	B2M	β 2 microglobulin
LIPH	lipase, member H (mPA-PLA1)	GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LIPI	lipase, member I (mPA-PLA1beta)	HMBS	hydroxymethylbilane synthase
PLA1A	phospholipase A1A	HPRT1	hypoxanthine phosphoribosyltransferase 1
PLA2G2A	secretory phospholipase A2	PGK1	phosphoglycerate kinase 1
PLA2G4A	cytosolic phospholipase A2	PPIA	peptidylprolyl isomerase A
PPAP2A	lipid phosphate phosphohydrolase 1	TFRC	transferrin receptor
PPAP2B	lipid phosphate phosphohydrolase 3	PTGIR	prostaglandin I2 receptor
PPAP2C	lipid phosphate phosphohydrolase 2	PDE4A	phosphodiesterase 4A
GNAS	Gαs	PDE4B	phosphodiesterase 4B
GNAI3	Gαi3	RAPGEF3	RAP guanine nucleotide exchange factor 3
GNAI2	Gαi2	RAPGEF4	RAP guanine nucleotide exchange factor 4
GNAI1	Gαi1	RXFP2	relaxin/insulin-like family peptide receptor 2
ADCY1	adenylyl cyclase 1	RXFP1	relaxin/insulin-like family peptide receptor 1
ADCY2	adenylyl cyclase 2	SPHK1	sphingosine kinase 1
ADCY3	adenylyl cyclase 3	SPHK2	sphingosine kinase 2

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:

95 °C	20 s	Template denaturation and enzyme initialisation	
95 °C	3 s	Denaturation	x 40 cycles
60 °C	30 s	Annealing and extension	

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 [^{35}S]GTPγS 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).

Gene target	Gene product	TaqMan® assay ID number
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs_02758991_g1
SOCS1	suppressor of cytokine signaling 1	Hs_00705164_s1
IL1A	interleukin 1, alpha	Hs_00174092_m1
MIF	macrophage migration inhibitory factor	Hs_00236988_g1
CCL11	chemokine (C-C motif) ligand 11	Hs_00237013_m1
LIPH	lipase, member H	Hs_00975890_m1
PPAP2C	phosphatidic acid phosphatase type 2C, LPP2	Hs_00186575_m1
PDE4B	phosphodiesterase 4B, cAMP-specific	Hs_00963643_m1
PDE4D	phosphodiesterase 4D, cAMP-specific	Hs_01579625_m1
SPHK2	sphingosine kinase 2	Hs_00219999_m1
ADCY8	adenylate cyclase 8	Hs_00181588_m1
RAPGEF3	Rap guanine nucleotide exchange factor (GEF) 3	Hs_00183449_m1
GNAS	adenylate cyclase-stimulating G alpha protein	Hs00255603_m1

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 [^{35}S]GTPγS binding assay buffer (10 mM HEPES, 100 mM NaCl, and 10 mM $MgCl_2$, pH 7.4) for 10 min at room temperature, before addition of 300 pM [^{35}S]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 $[Ca^{2+}]_i$ 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 % CO_2. 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 Ca^{2+}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 $MgSO_4.7\ H_2O$, 1.2 mM KH_2PO_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 % Na_2CO_3, 0.4 % NaOH:1 % $CuSO_4$: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 Ca^{2+} or Mg^{2+}) 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 % CO_2 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:

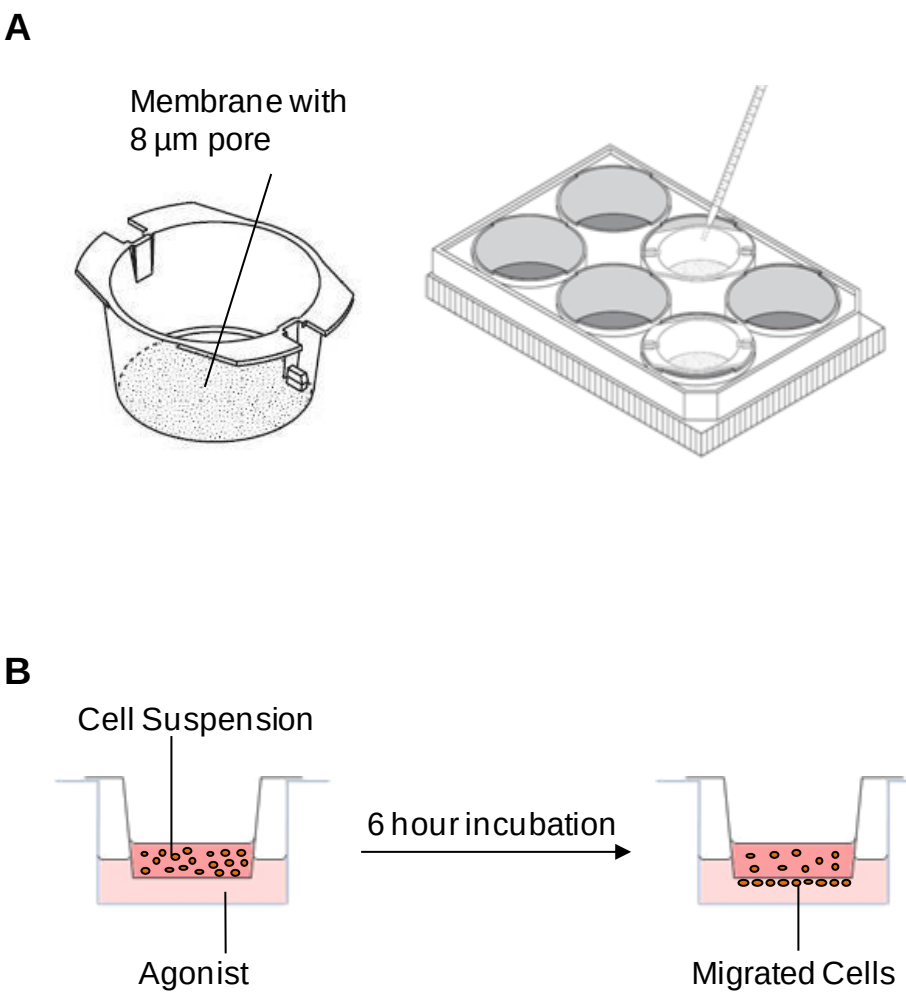


Figure 2.2

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 % CO_2 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

Buffer component	Final concentration
β-Glycerophosphate, pH 7.4	10 mM
EDTA, pH 8.0	1 mM
EGTA	1 mM
Tris-HCl, pH 7.5	50 mM
Benzamidine	1 mM
Sodium orthovanadate	1 mM
PMSF	0.2 mM
Pepstatin A	1.5 µM
Leupeptin	2.3 µM
β-Mercaptoethanol	0.1 %
Triton X-100	1 %
Sodium Fluoride	50 mM

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. 4 x sample buffer (see Table 2.9 for sample buffer composition) was then added to the sample to yield a 1 x 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.

Buffer	Composition	
Sample Buffer	62.5 mM tris (pH 6.8), 1 % SDS, 0.001 % bromophenol blue, 10 % glycerol, 2.5 % β-mercaptoethanol	
10 % polyacrylamide gels	Running gel:	10 % acrylamide, 375 mM tris (pH 8.8), 0.1 % SDS, 0.1 % APS, 0.0004 % TEMED
	Stacking gel:	5 % acrylamide, 125 mM tris (pH 6.8), 0.1 % SDS, 0.1 % APS, 0.001 % TEMED
Running buffer	25 mM tris, 192 mM glycine, 0.1 % SDS	
TBS-T	20 mM tris, 137 mM NaCl, 0.1 % Tween® 20, pH 7.6	
Transfer buffer	25 mM tris, 192 mM glycine, 0.02 % SDS, 20 % methanol	

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_H2 cytokines so are notable in asthma research. IL-4 and IL-13 are closely related interleukins which are important in driving T_H2 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_H2 development so has clear significance for allergic disease. IL-10 is a regulatory cytokine which is secreted by T_H2 and T_{reg} cells and may have disrupted function in airways disease, while IL-18 induces IFN-γ production and promotes a T_H1 response (Murphy, 2012). Genes for the pro-inflammatory cytokine, TNFα 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 NFκB-1, -2 and -3, interferon-γ and nitric oxide synthase 2 (NOS2).

Several chemokines from the CCL, CXCL and CX_3CL 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), *NRP1* and *-2*, *SEMA6C*, *SEMA7A* (Smith *et al.*, 2011), *PIM1* (Shin *et al.*, 2012), *PLAUR*, *SERPINB2*, *SERPINB1* (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 $A_{1/2}$ ($PLA_{1/2}$; 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 $PLA_{1/2}$ 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 PLA_1s, mPA-PLA_1-α and -β respectively, while *PLA2G2A* and *PLA2G4A* encode the secretory PLA_2 group IIA ($sPLA_2$-IIA) and the cytosolic PLA_2 ($cPLA_2$). The phosphatidylserine (PS)

selective PLA_1, PS-PLA_1 (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.*

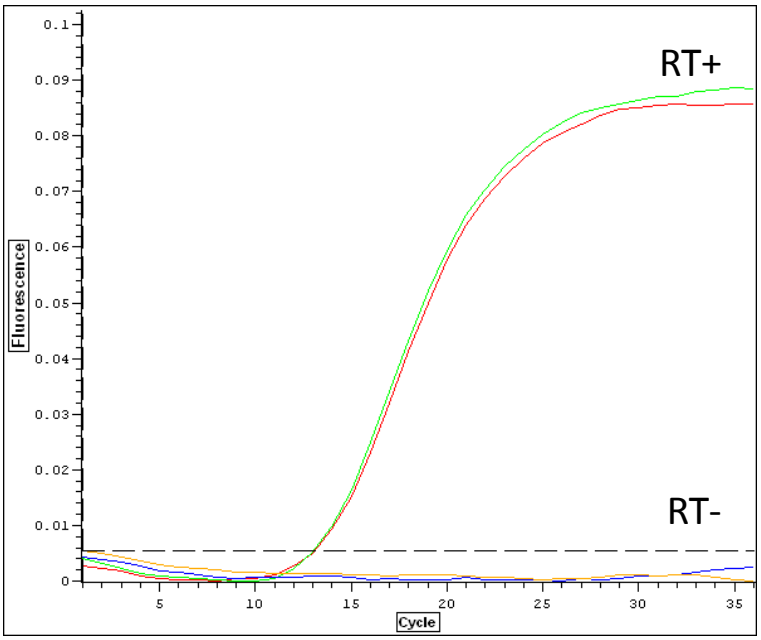
Genomic DNA contamination was assessed by performing RT-qPCR experiments on all RT+ and RT- cDNA samples using primers for 18S rRNA which would allow amplification of the rat or human sequence. Since 18S rRNA is abundant in all mammalian cell types, this would provide a sensitive test for contamination. cDNA samples were generated from RNA isolated from clinically characterised cultured hASM cells from control or asthmatic donors. While a DNAse 1 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 TLDAs*

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.

A.



B.

	Proportion of samples with 18S amplification	C_t	
		Mean	± s.e.m.
RT+	26 / 26	12.18	± 0.14
RT-	3 / 26	33.84	± 0.12

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. C_t values for RT+ and RT- samples are significantly different as assessed by *t*-test, $p < 0.0001$.

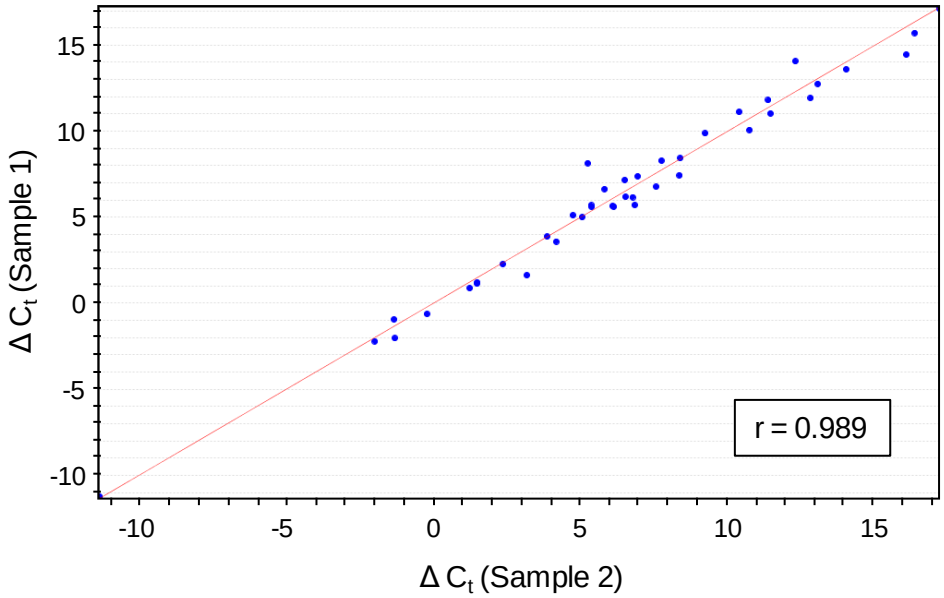


Figure 3.2

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_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_t values for *RN18S1* on the inflammation TLDA (samples A109 and A169) and one slightly low C_t for *GAPDH* on the LPA TLDA (sample A109). The outlying results can be easily identified on the amplification plots (Figure 3.3**A** and Figure 3.4**C**) 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_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_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 $p = 0.05$ ($= -\log_{10}(1.3)$) 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_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

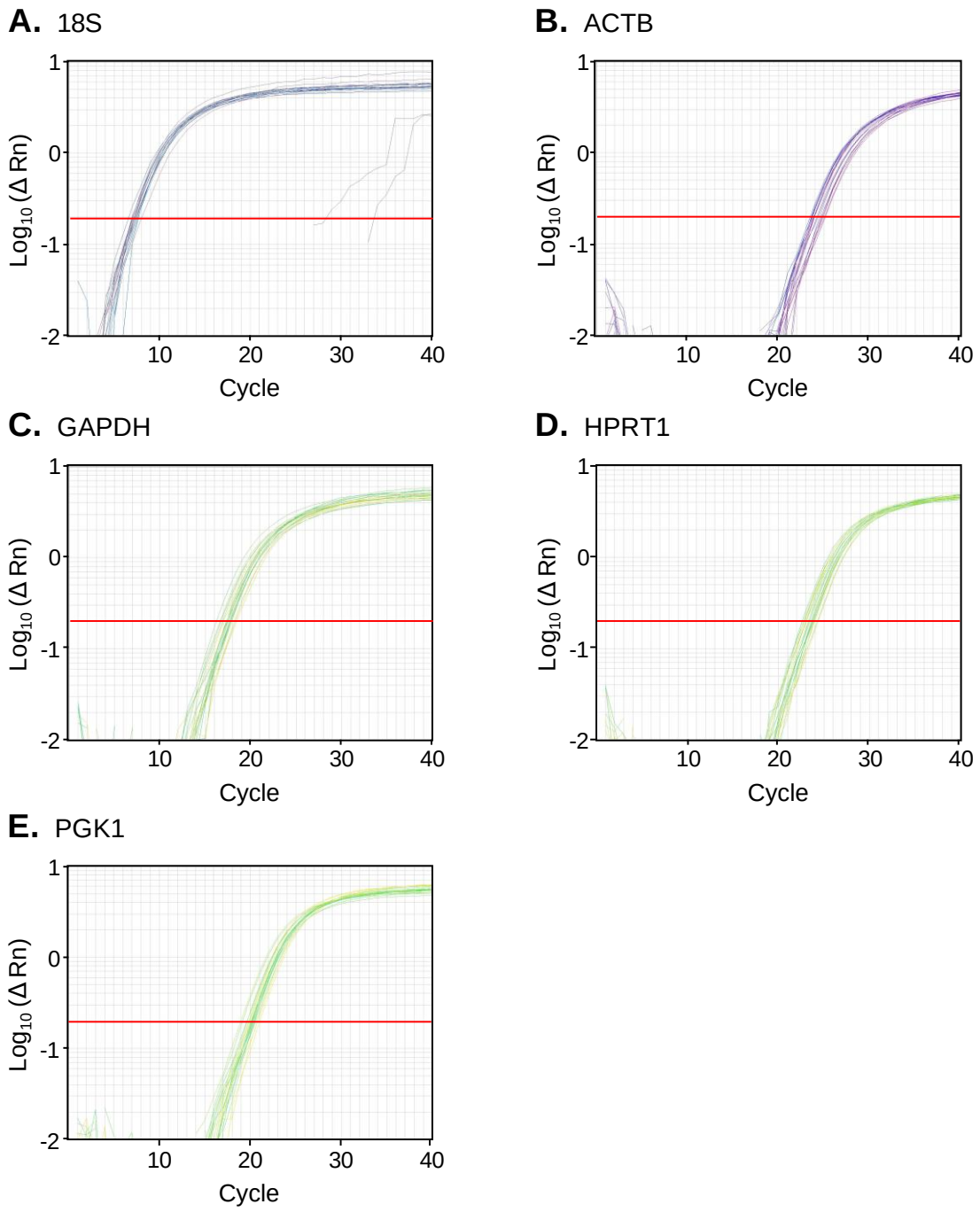


Figure 3.3

Amplification plots for the candidate reference genes for inflammation TLDAs. 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 20 cDNA samples used are overlaid for each gene target. **A**–**E** show amplification plots for *RN18S1*, *ACTB*, *GAPDH*, *HPRT1* and *PGK1*, respectively.

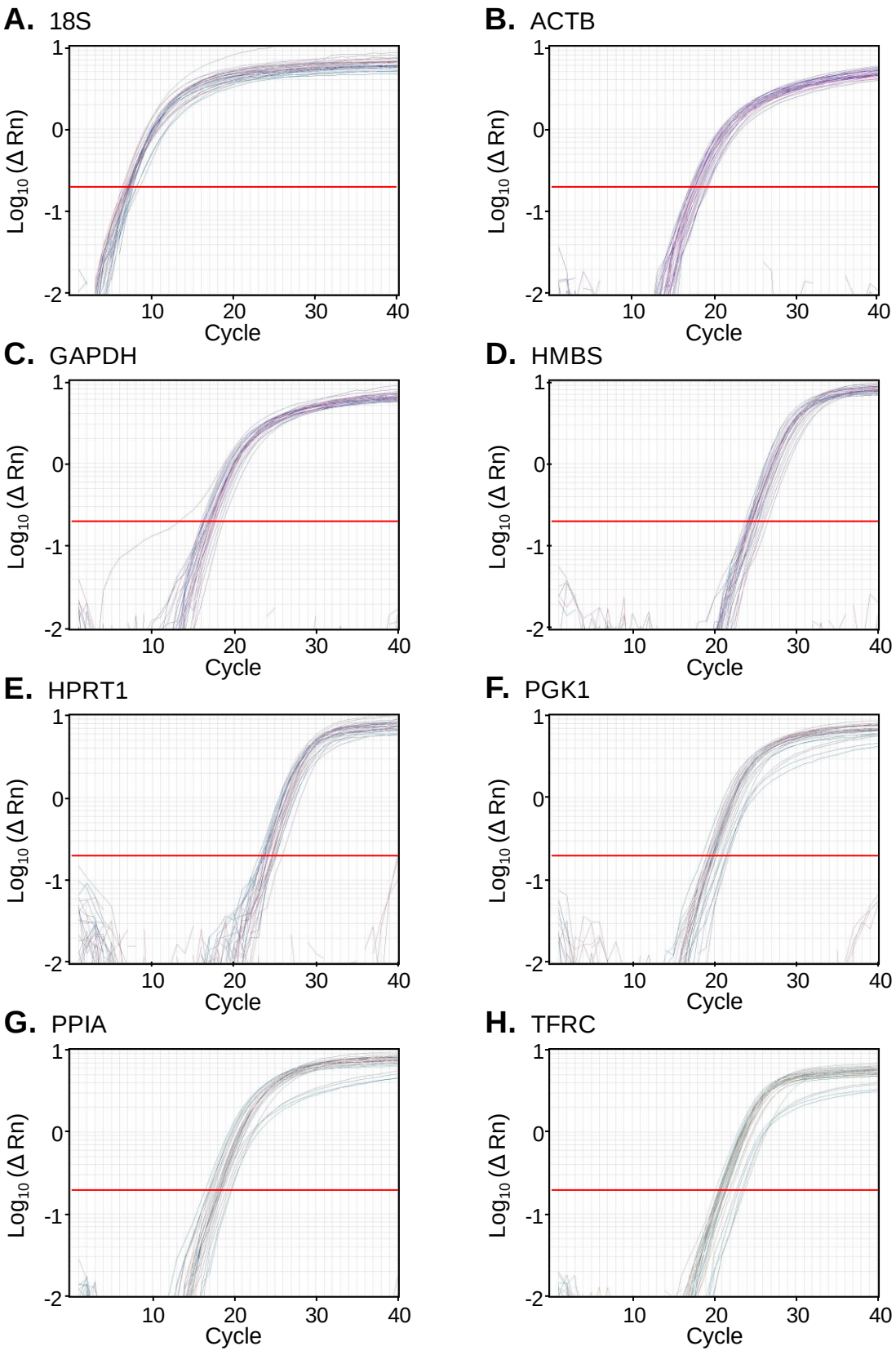

A. 18S
B. ACTB
C. GAPDH
D. HMBS
E. HPRT1
F. PGK1
G. PPIA
H. TFRC
Log10 (Δ Rn)
Cycle
1
0
-1
-2
10
20
30
40

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.

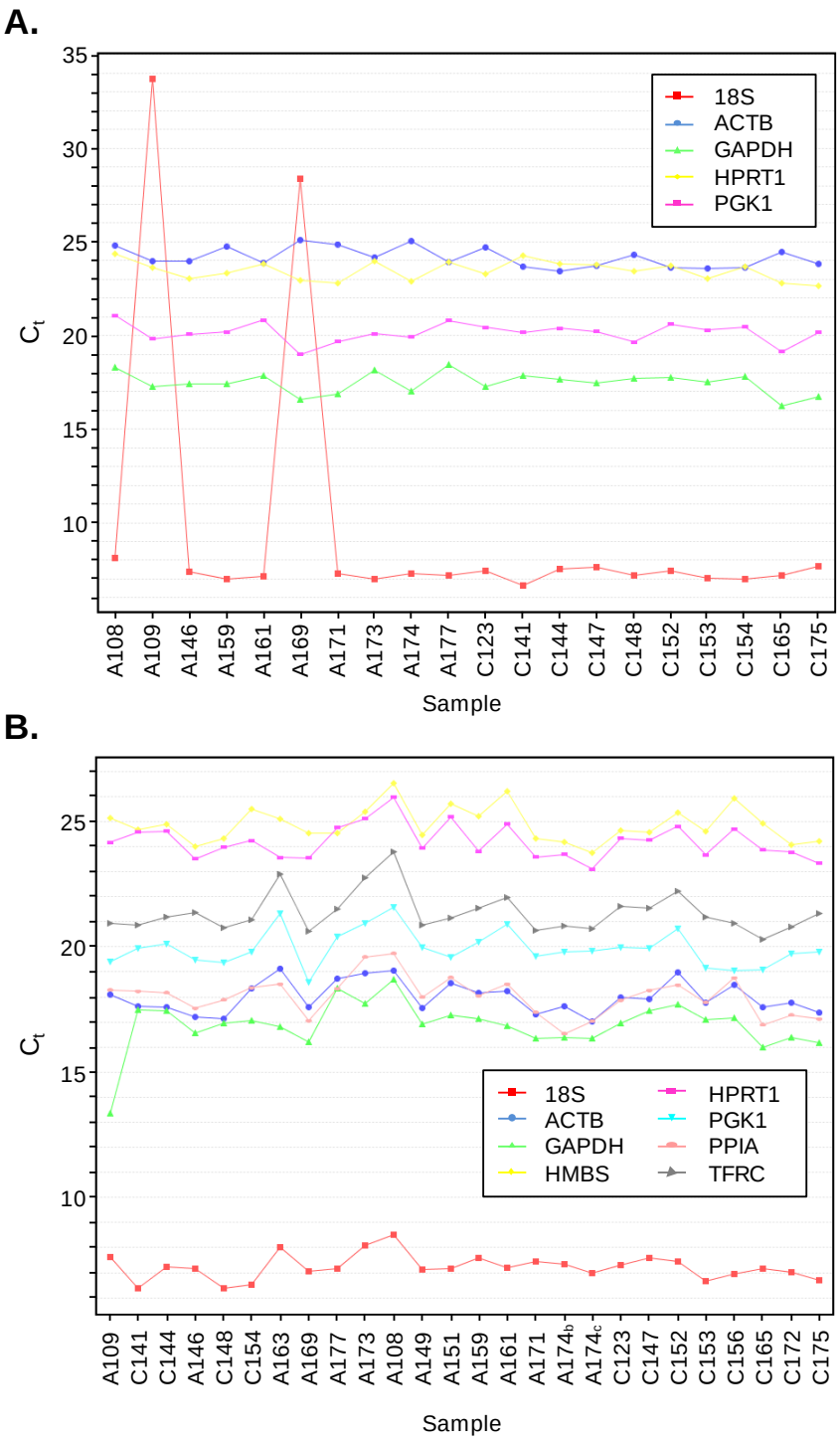


Figure 3.5

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.

A.

Candidate reference gene	Gene stability score	C_t	
		Mean	± s.e.m.
18S	7.466	9.66	± 1.65
ACTB	2.491	24.17	± 0.12
GAPDH	2.286	17.47	± 0.13
HPRT1	2.262	23.46	± 0.12
PGK1	2.327	20.14	± 0.12

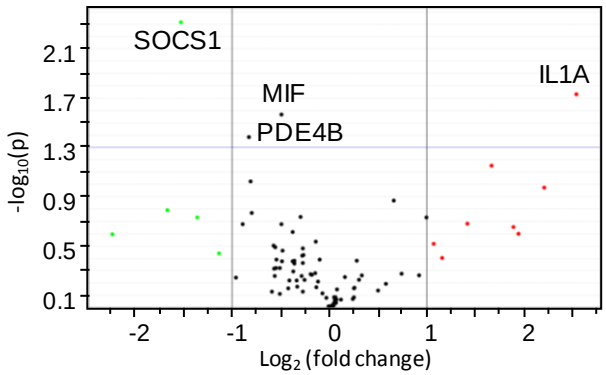
B.

Candidate reference gene	Gene stability score	C_t	
		Mean	± s.e.m.
18S	0.671	7.23	± 0.10
ACTB	0.572	17.99	± 0.12
GAPDH	0.914	16.88	± 0.19
HMBS	0.646	24.88	± 0.14
HPRT1	0.608	24.19	± 0.13
PGK1	0.637	19.94	± 0.14
PPIA	0.604	18.02	± 0.15
TFRC	0.620	21.36	± 0.16

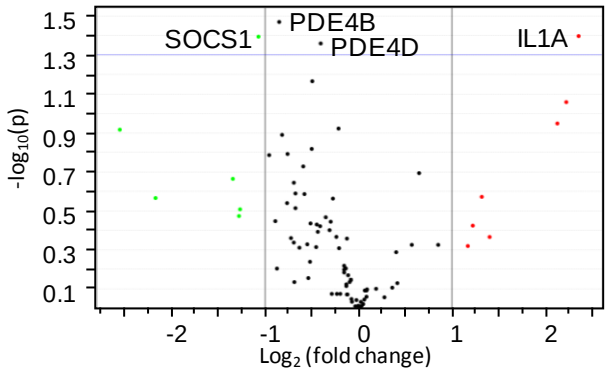
Table 3.1

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).

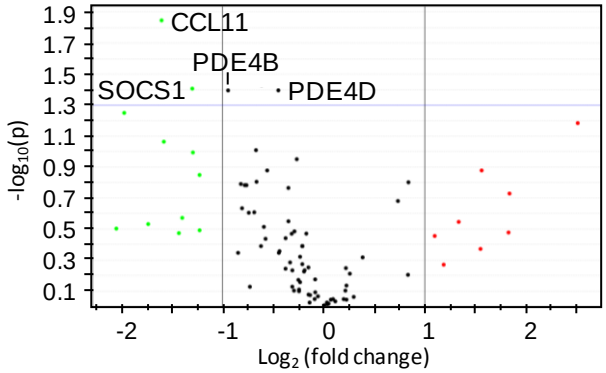
A. All asthmatics, maximum values included



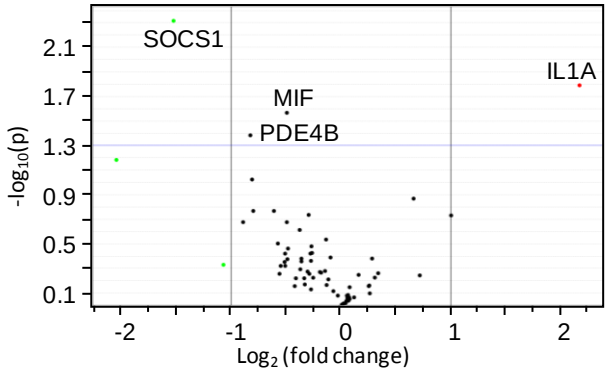
B. All asthmatics, maximum values excluded



C. Severe and moderate asthmatics, maximum values included



D. Severe and moderate asthmatics, maximum values excluded



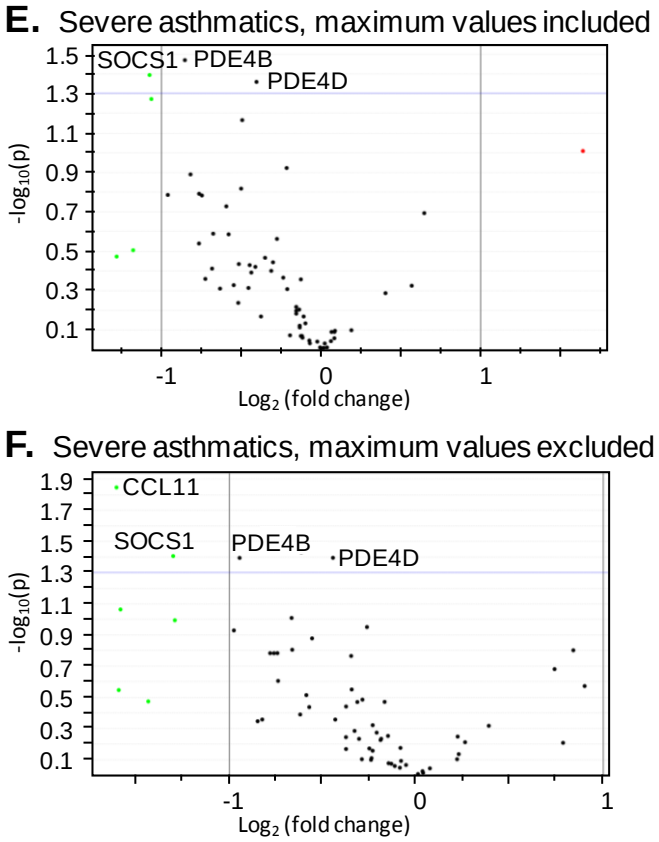
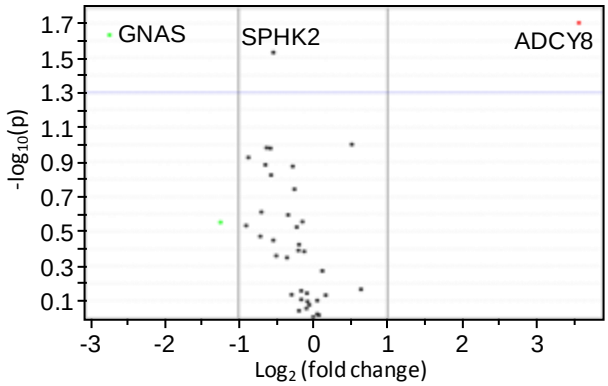


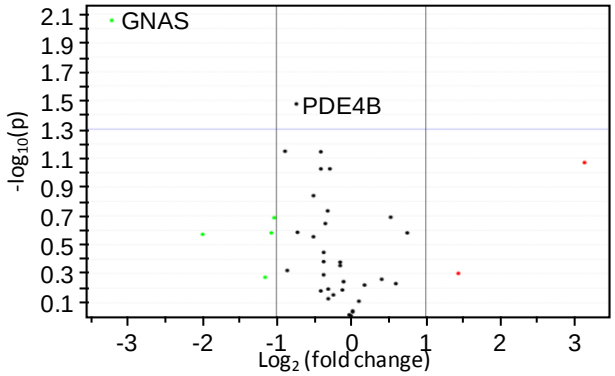
Figure 3.6

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.

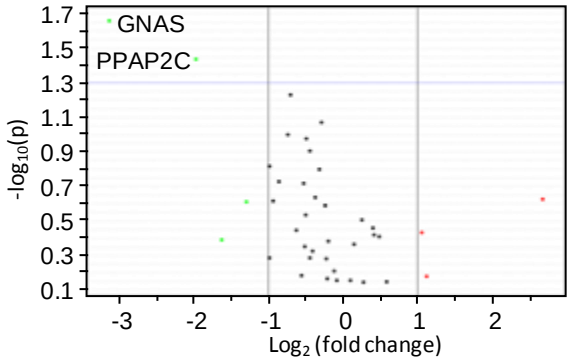
A. All asthmatics, maximum values included



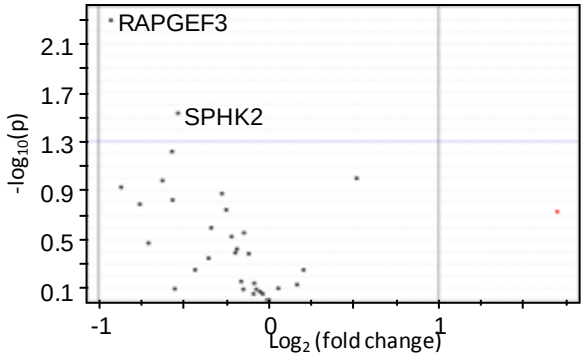
B. All asthmatics, maximum values excluded



C. Severe and moderate asthmatics, maximum values included



D. Severe and moderate asthmatics, maximum values excluded



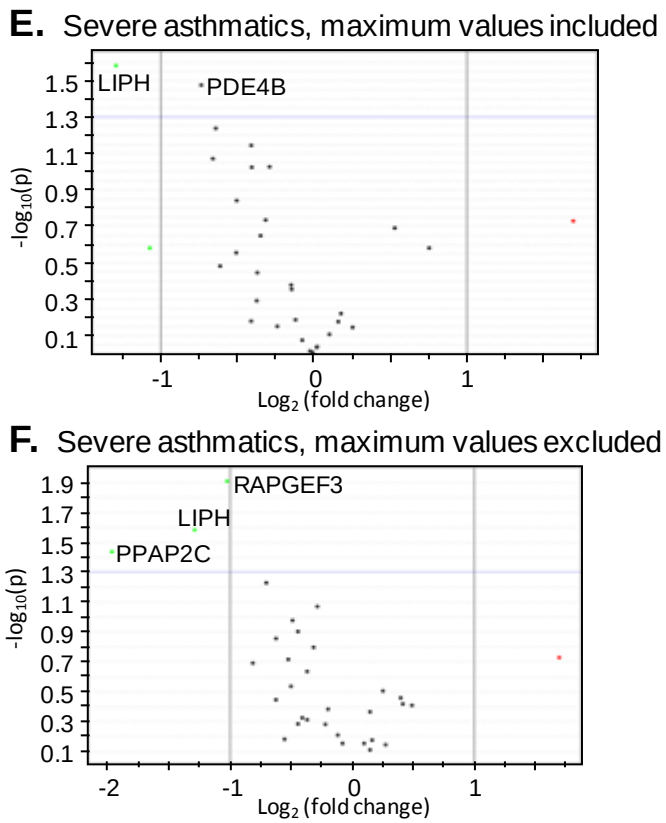


Figure 3.7

Volcano plots illustrating the variation in C_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 $\pm \log_2(1)$ 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.

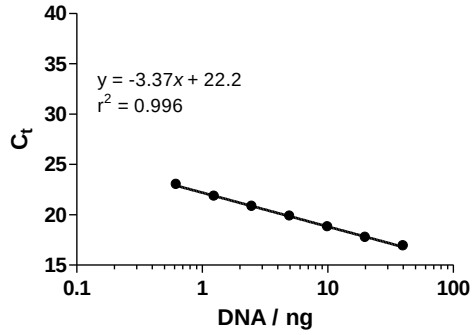
values are included or not, but only where the asthmatic group is limited to donors exhibiting severe disease.

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

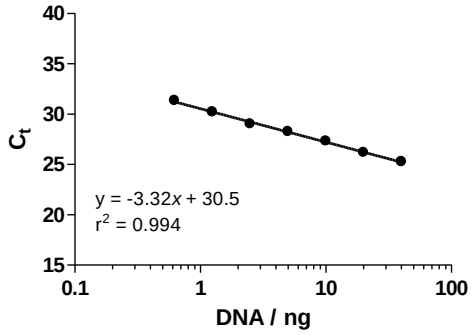
Expression levels of a number of genes of interest identified from the TLDA experiments described above were investigated using samples derived from low-passage hASM cells from control and asthmatic donors by RT-qPCR and the standard curve method of analysis. Standard curves, prepared using a range of 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 3.8**A**), indicating relatively high abundance of *GAPDH* transcript in the calibrator sample, whereas *LIPH* (Figure 3.8**F**) 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 3.8**K**-**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

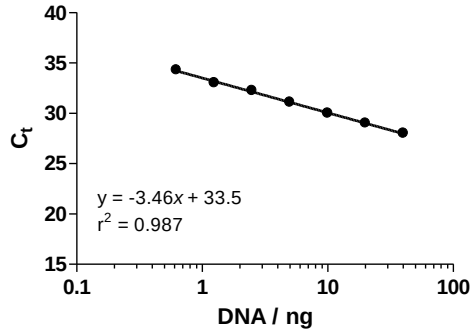
A. GAPDH



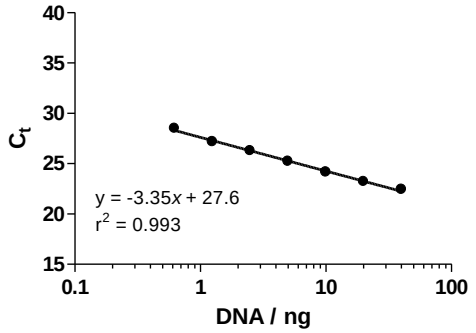
B. SOCS1



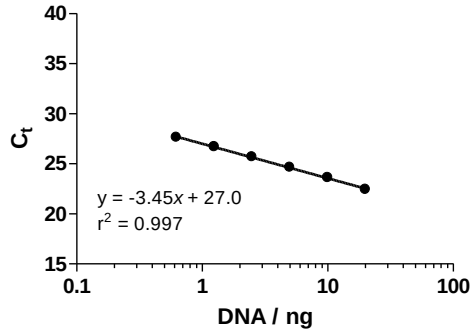
C. IL1A



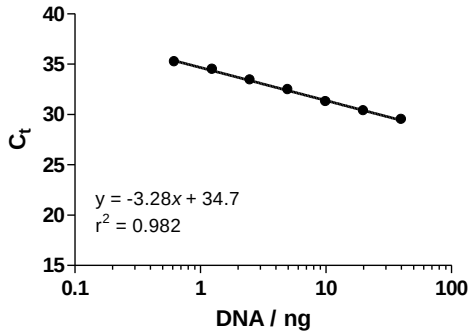
D. MIF



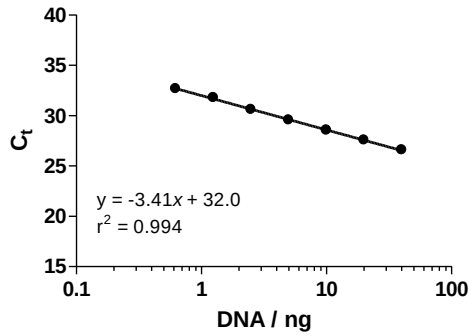
E. CCL11



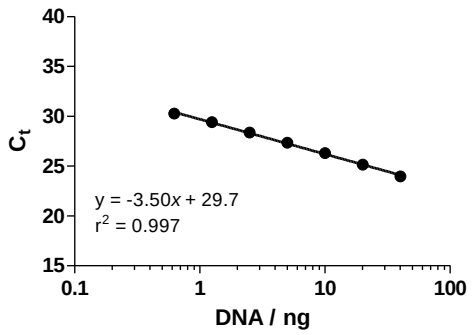
F. LIPH



G. PPAP2C



H. PDE4B



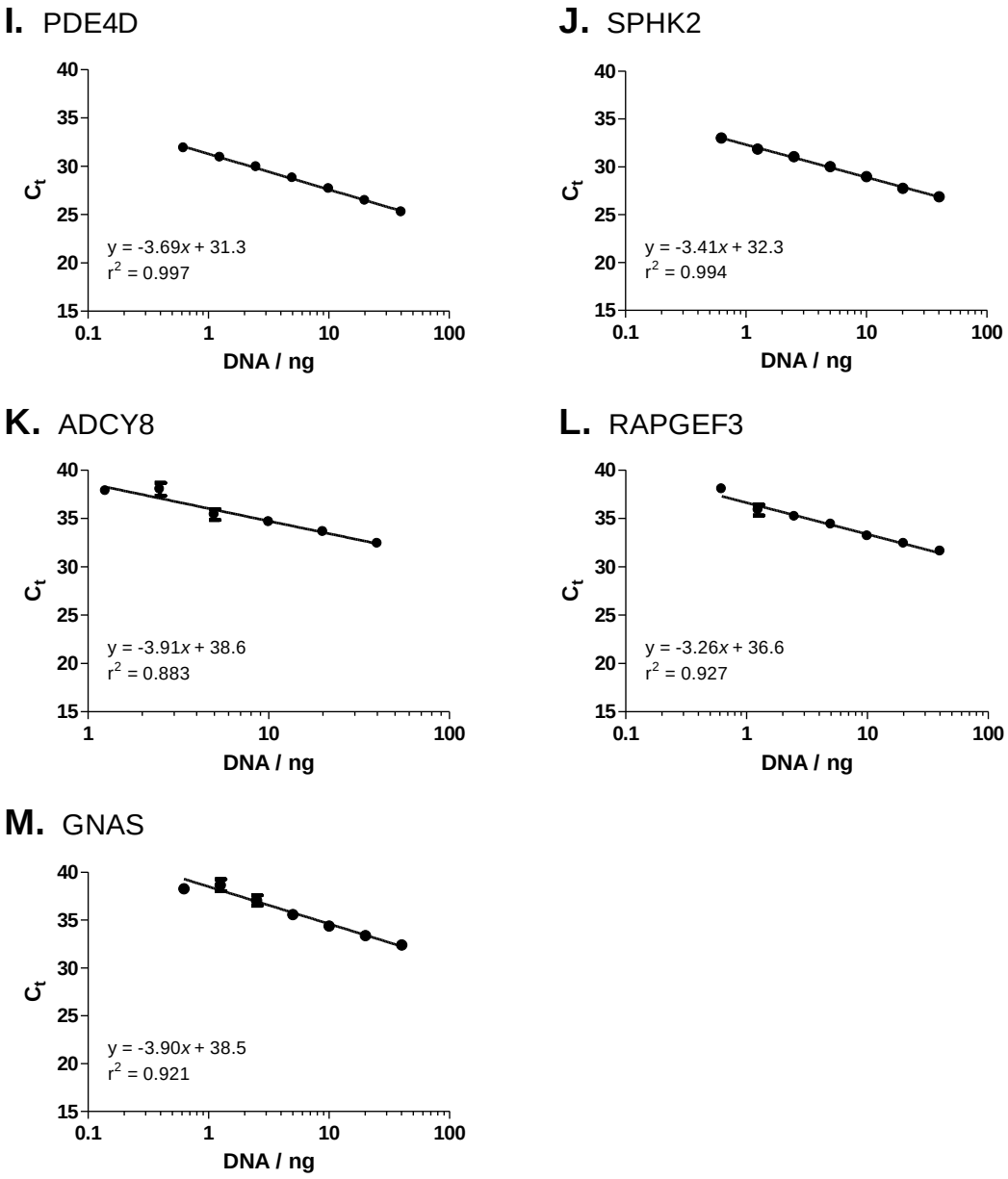
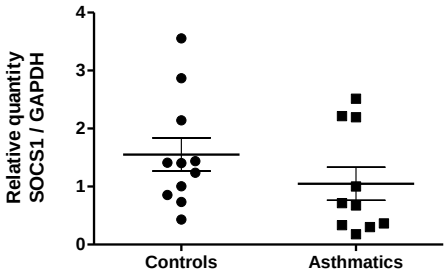


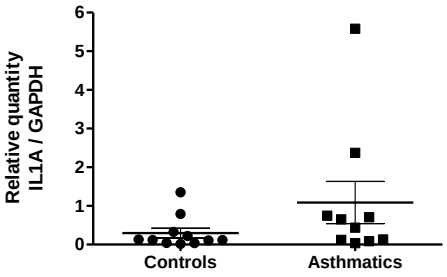
Figure 3.8

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_t value (± s.e.m.) against total amount of input DNA in ng on a logarithmic scale. Line equations and R^2 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.

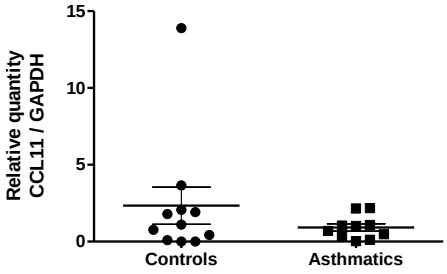
A. SOCS1



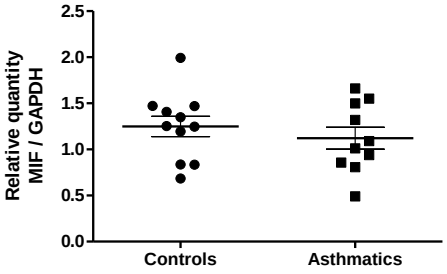
B. IL1A



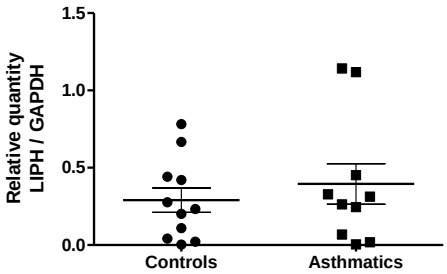
C. MIF



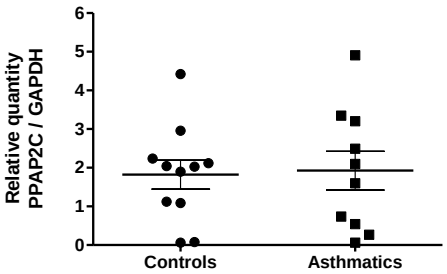
D. CCL11



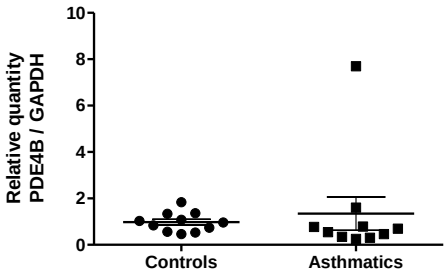
E. LIPH



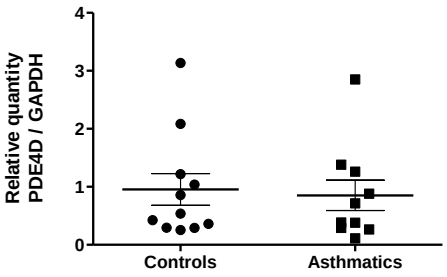
F. PPAP2C



G. PDE4B



H. PDE4D



I. SPHK2

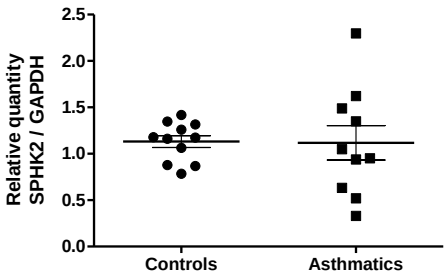


Figure 3.9

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 ± 0.126 and 0.526 ± 0.103 for controls and asthmatics, respectively (Figure 3.10**A**). 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 ± 0.376 to 0.402 ± 0.148 ($p = 0.046$; Figure 3.10**B**). 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 3.10**C**).

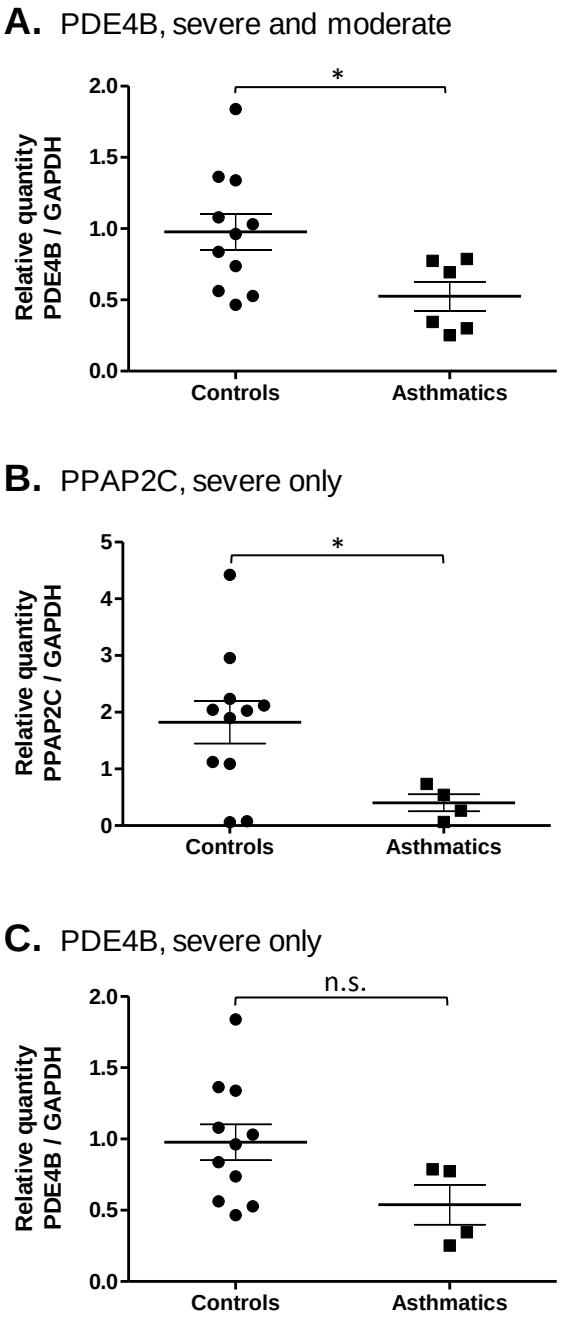


Figure 3.10

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 $p \leq 0.05$

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 (ΔC_t 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 ($\geq$ 2-fold) and/or significant ($p \leq 0.05$) 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, C_t 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 β_2-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 hyper-responsiveness 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 Ca^{2+} 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 LPA_1 and LPA_2 receptors with the exception of 12:0, which was not active at any of the receptors, while LPA_3 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 [^{35}S]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}S]GTPγS 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}S]GTPγS 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 ((2*S*)-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}S]GTPγS 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}S]GTPγS 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.*,

2004). 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}S]GTPγS binding assay (Heasley *et al.*, 2004) and 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 LPA_5 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}S]GTPγS 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 α subunit of the G protein Gαβγ trimer on agonist binding to the receptor and consequent dissociation of activated Gα-GTP from Gβγ subunits, which then effect modulations of downstream signalling events. The intrinsic GTPase activity of Gα allows a return to the GDP-bound form and re-association of Gα-GDP with Gβγ subunits. GTPγS is a hydrolysis-resistant analogue of GTP that is very poorly susceptible to the GTPase activity of the Gα subunit and so an accumulation of GTPγS-bound Gα subunits in cell membranes can be measured. The use of [^{35}S]GTPγS 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, $G\alpha_{15,}$ to permit coupling to the PLC/IP_3/Ca^{2+} 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 LPA_1, LPA_2 and LPA_3 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}S]GTPγS binding assay

A [^{35}S]GTPγS 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}S]GTPγS concentration.

[^{35}S]GTPγS binding assay time-courses

It was important to ascertain an appropriate incubation time which allows for adequate accumulation of [^{35}S]GTPγS bound Gα in the membrane for detection, without allowing too much basal [^{35}S]GTPγS binding to occur. Time-course experiments were performed in which RH7777-LPA$_1$ cell membranes were stimulated with different LPA concentrations and total [^{35}S]GTPγS 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}S]GTPγS 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

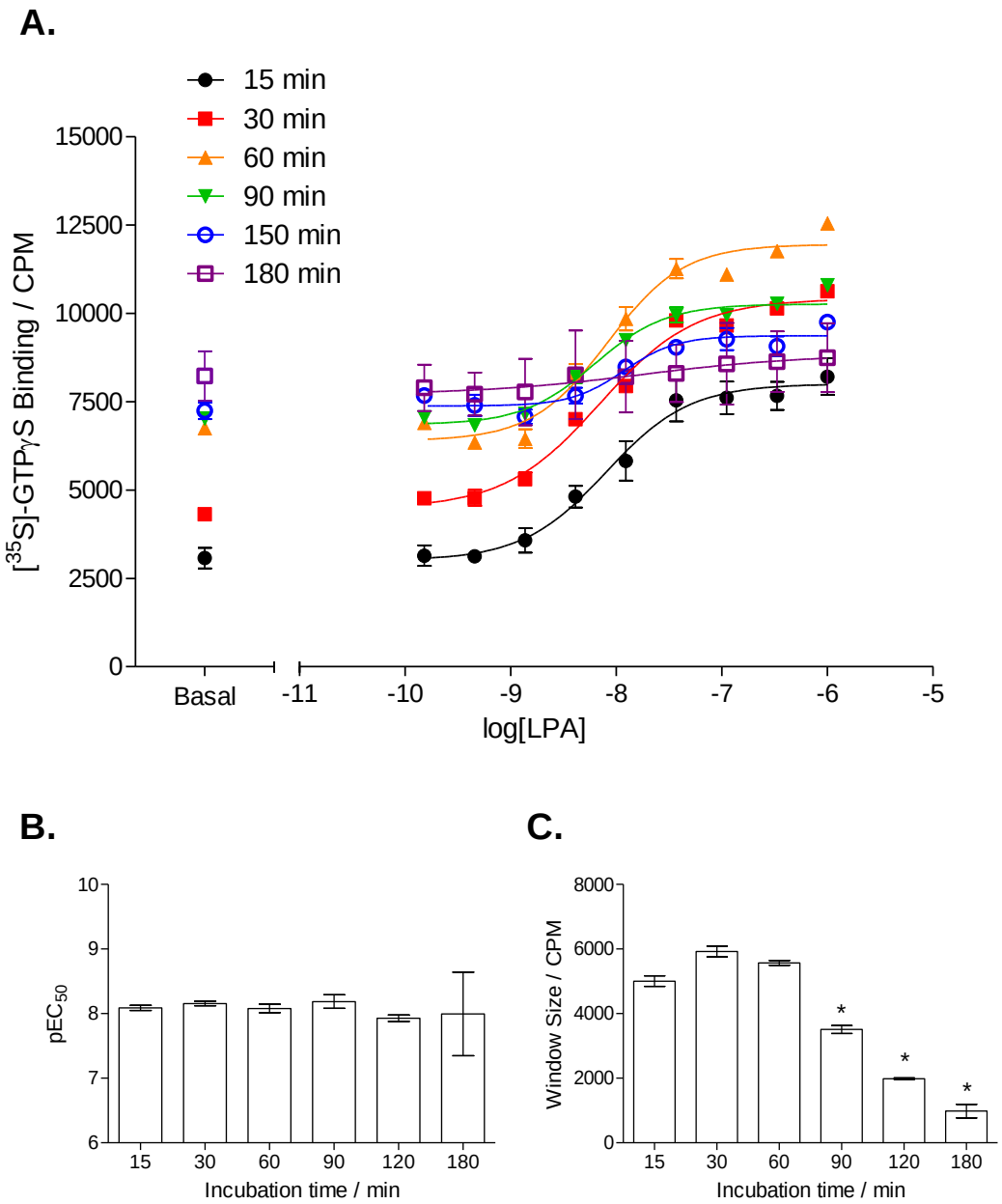


Figure 4.1

[^{35}S]GTPγS binding assay time-courses. **A**, RH7777-LPA$_1$ membranes were stimulated with various LPA concentrations and incubated with [^{35}S]GTPγS 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, $p \leq 0.01$.

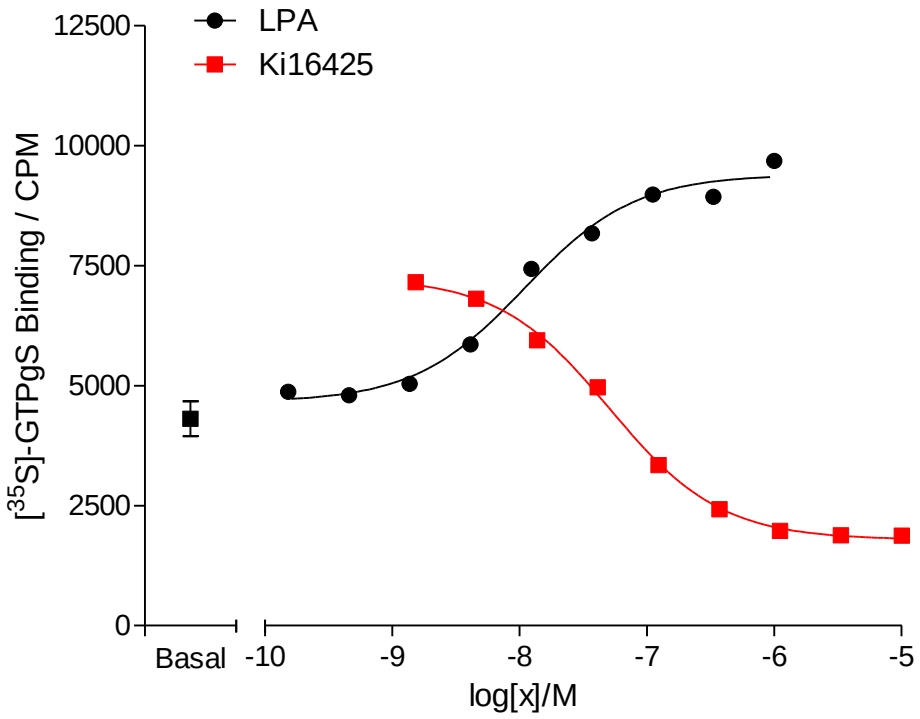


Figure 4.2

LPA concentration-response curve and concentration-dependent inhibitory effects of the LPA receptor antagonist, Ki16425, for [^{35}S]GTPγS 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 ± 0.06 and 7.30 ± 0.02 for LPA and Ki16425, respectively.

experiment, experiments were performed wherein RH7777-LPA_1 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 4.3**A**), nor did the presence of sodium orthovanadate affect the results (Figure 4.3**B**). 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 [^{35}S]GTPγS assay is typically performed in the presence of GDP, which serves to occupy 'empty' Gα subunits and to compete with [^{35}S]GTPγS for Gα binding and thus reduce the levels of basal [^{35}S]GTPγS binding, allowing optimal measurement of agonist-induced [^{35}S]GTPγS binding. The optimum GDP concentration should be determined since this is system-dependent, as is the optimal concentration of [^{35}S]GTPγS. The concentration of membrane protein required to achieve optimal [^{35}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 [^{35}S]GTPγS (Figure 4.4). As expected, increasing GDP concentration reduced levels of [^{35}S]GTPγS binding, without affecting EC_{50} values. While the presence of 10 µM GDP resulted in the lowest basal levels of [^{35}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 [^{35}S]GTPγS increased the magnitude of response, without affecting EC_{50} values. A membrane concentration of 20 µg per well and [^{35}S]GTPγS concentration of 300 pM were chosen to give a good window of activation. These assay conditions were applied in all further experiments.

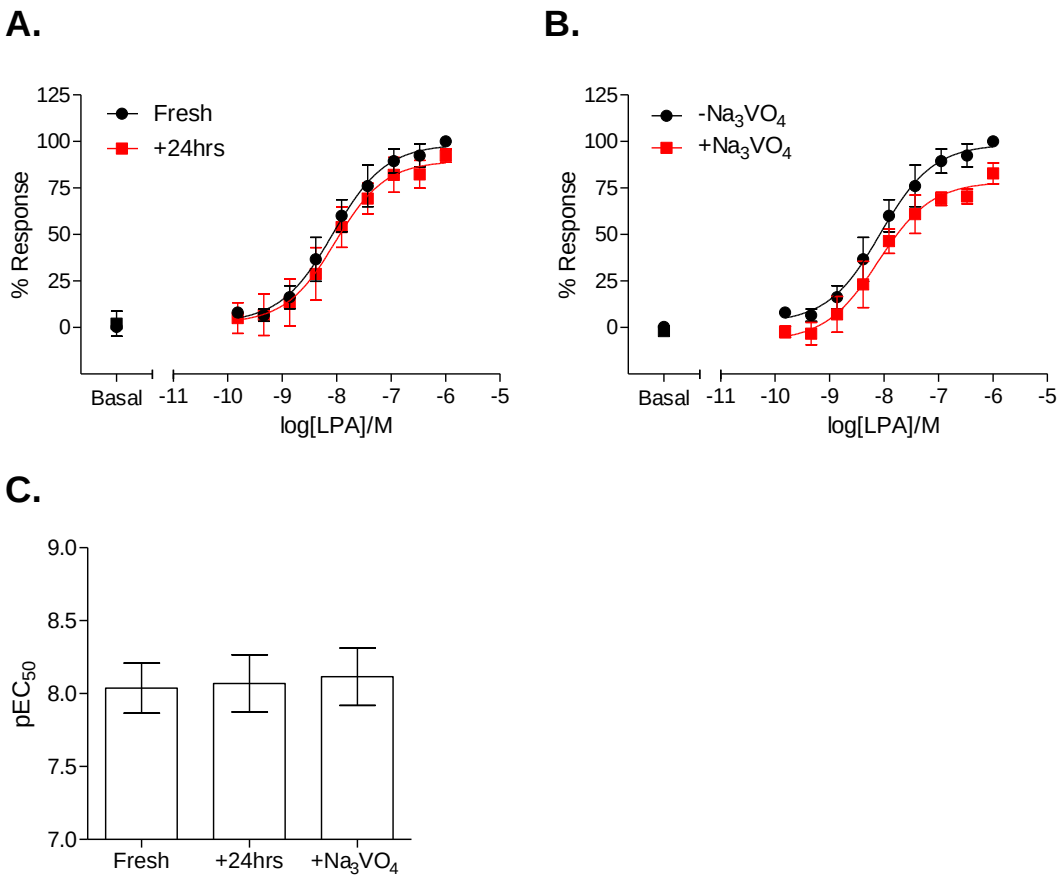


Figure 4.3

Assessment of LPA stability under the employed assay conditions. **A**, RH7777-LPA_1 membranes were stimulated with LPA that had been freshly prepared, or that had been left at room temperature for 24 h. **B**, RH7777-LPA_1 membranes were stimulated with freshly prepared LPA in the presence or absence of sodium orthovanadate at a final concentration of 100 µM. **C**, pEC_{50} values obtained under the various conditions: no statistically significant differences were observed.

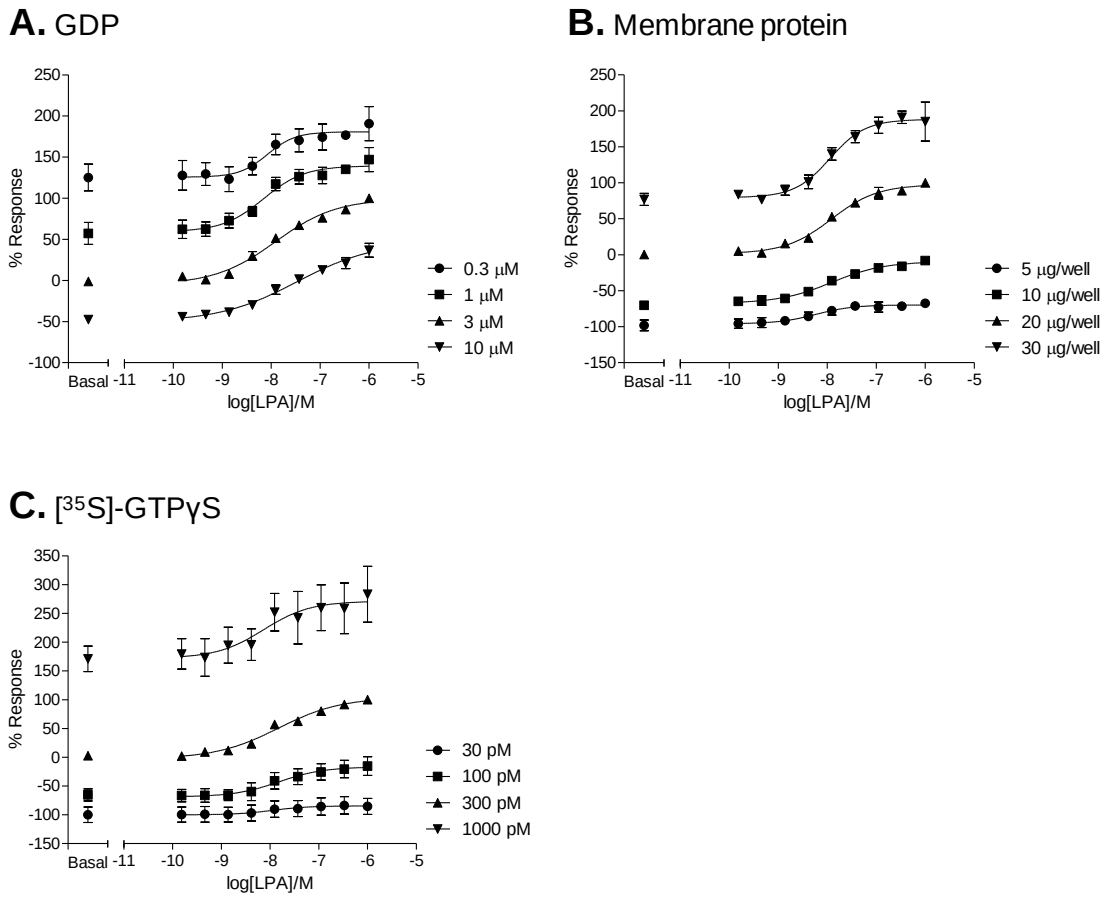


Figure 4.4

Optimisation of assay variables. LPA concentration-response curves generated from [^{35}S]GTPγS binding assays performed using various GDP (**A**), membrane protein (**B**) and [^{35}S]GTPγS (**C**) final concentrations as indicated. EC_{50} 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 [^{35}S]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-LPA_1 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 EC_{50}, were not altered significantly by the presence of either solvent (Figure 4.5).

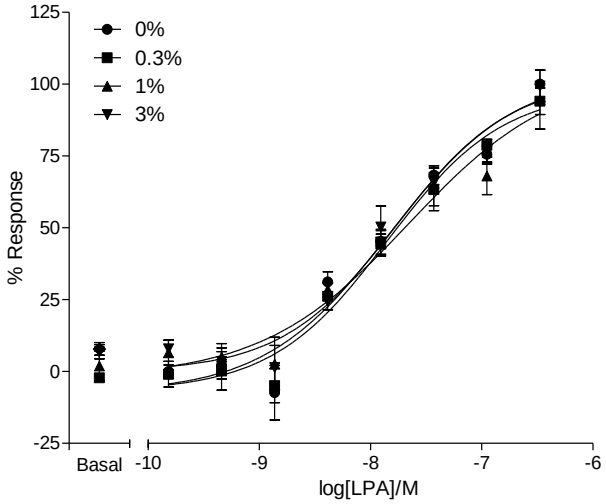
4.2.2. Effect of pertussis toxin treatment on [^{35}S]GTPγS binding in RH7777-LPA_1 membranes

RH7777-wild-type membranes and RH7777-LPA_1 membranes derived from control or PTx pre-treated RH7777-LPA_1 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 [^{35}S]GTPγS-bound $G\alpha_i$ to the observed response from RH7777-LPA_1 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 [^{35}S]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 $G\alpha_i$ mediates essentially all LPA-induced [^{35}S]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 [^{35}S]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 LPA_1 receptor was tested (Figure 4.7). The LPA variants tested exhibited a range of potencies at RH7777-LPA_1 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



B. Ethanol tolerance

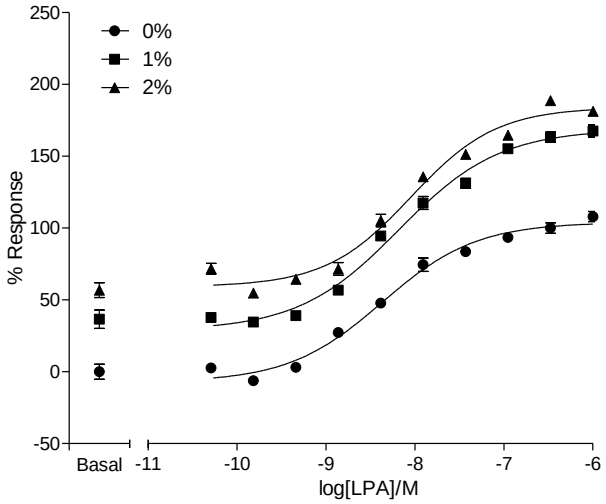


Figure 4.5

DMSO and ethanol tolerances of the [^{35}S]GTPγS binding assay. LPA concentration-response curves for [^{35}S]GTPγS binding assays performed in the presence of various final assay concentrations of DMSO (**A**), or ethanol (**B**). EC_{50} values or window sizes did not differ statistically significantly under any observed experimental conditions.

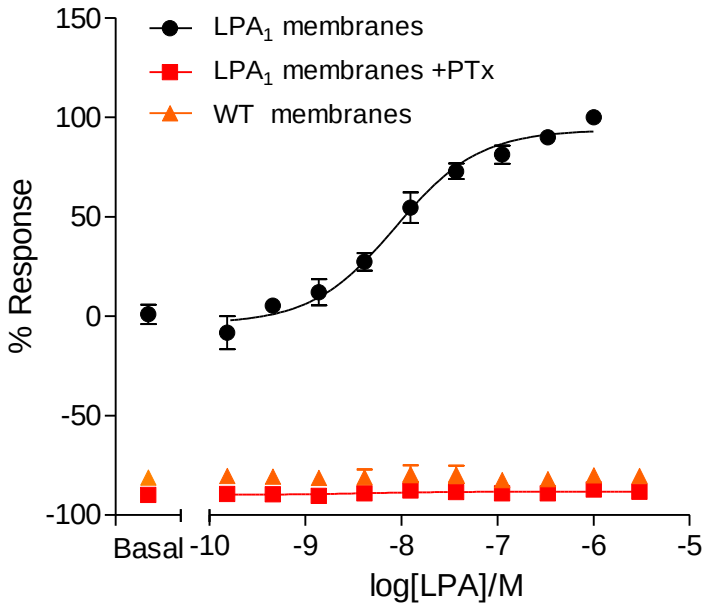


Figure 4.6

Effect of pertussis toxin (PTx) pre-treatment on LPA responses of RH7777-LPA_1 (LPA_1) and RH7777 wild-type (WT) membranes. Untreated or PTx pre-treated RH7777-LPA_1 membranes and untreated RH7777 wild-type membranes were stimulated with the various LPA concentrations indicated. Basal levels of [^{35}S]GTPγS binding were 5730 ± 620, 1237 ± 178 and 1668 ± 181 CPM for untreated LPA_1 membranes, LPA_1 membranes treated with PTx and wild-type membranes, respectively.

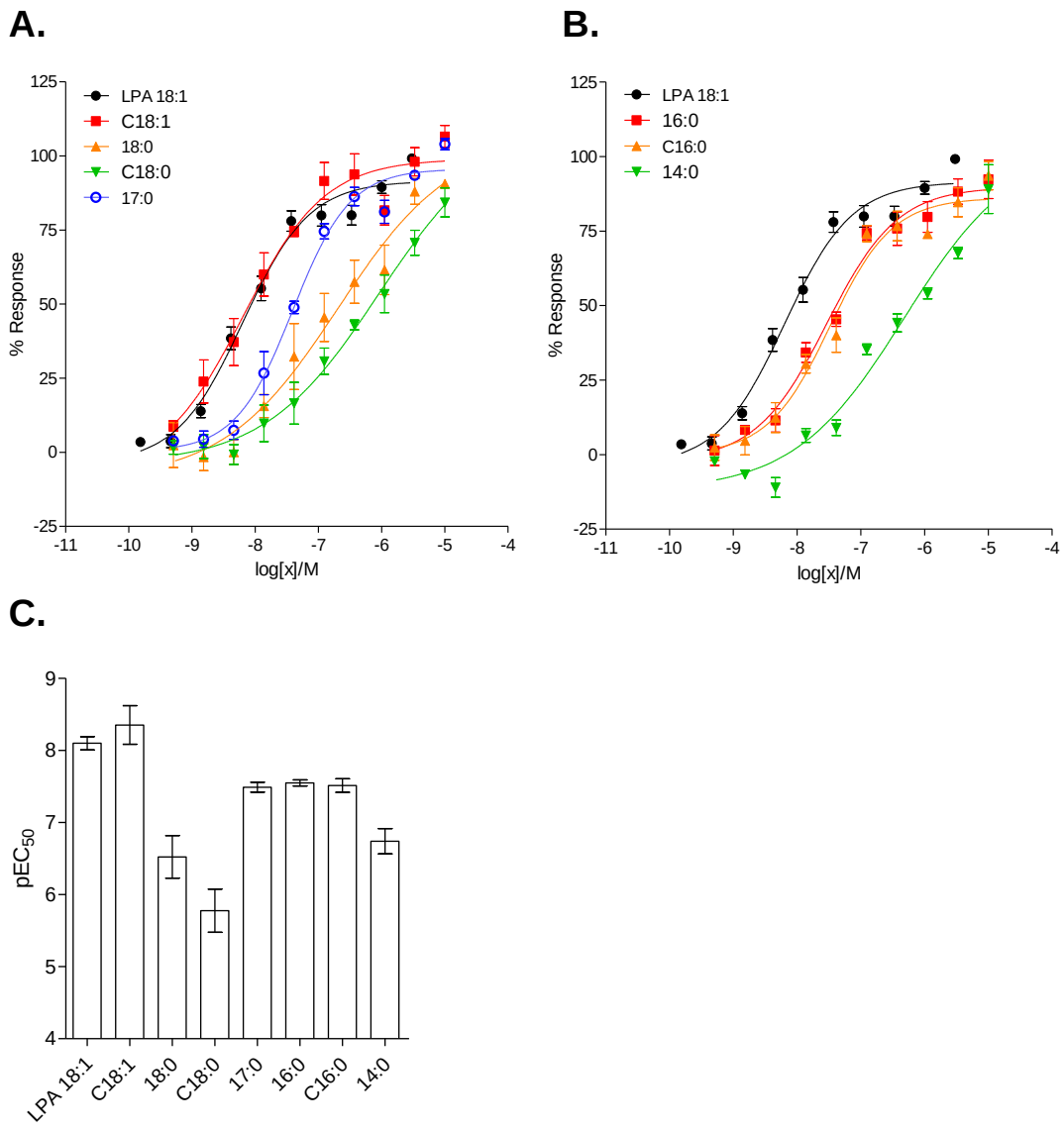


Figure 4.7

[^{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**.

(Santos *et al.*, 2000). 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}S]GTPγS 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}S]GTPγS 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}S]GTPγS 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

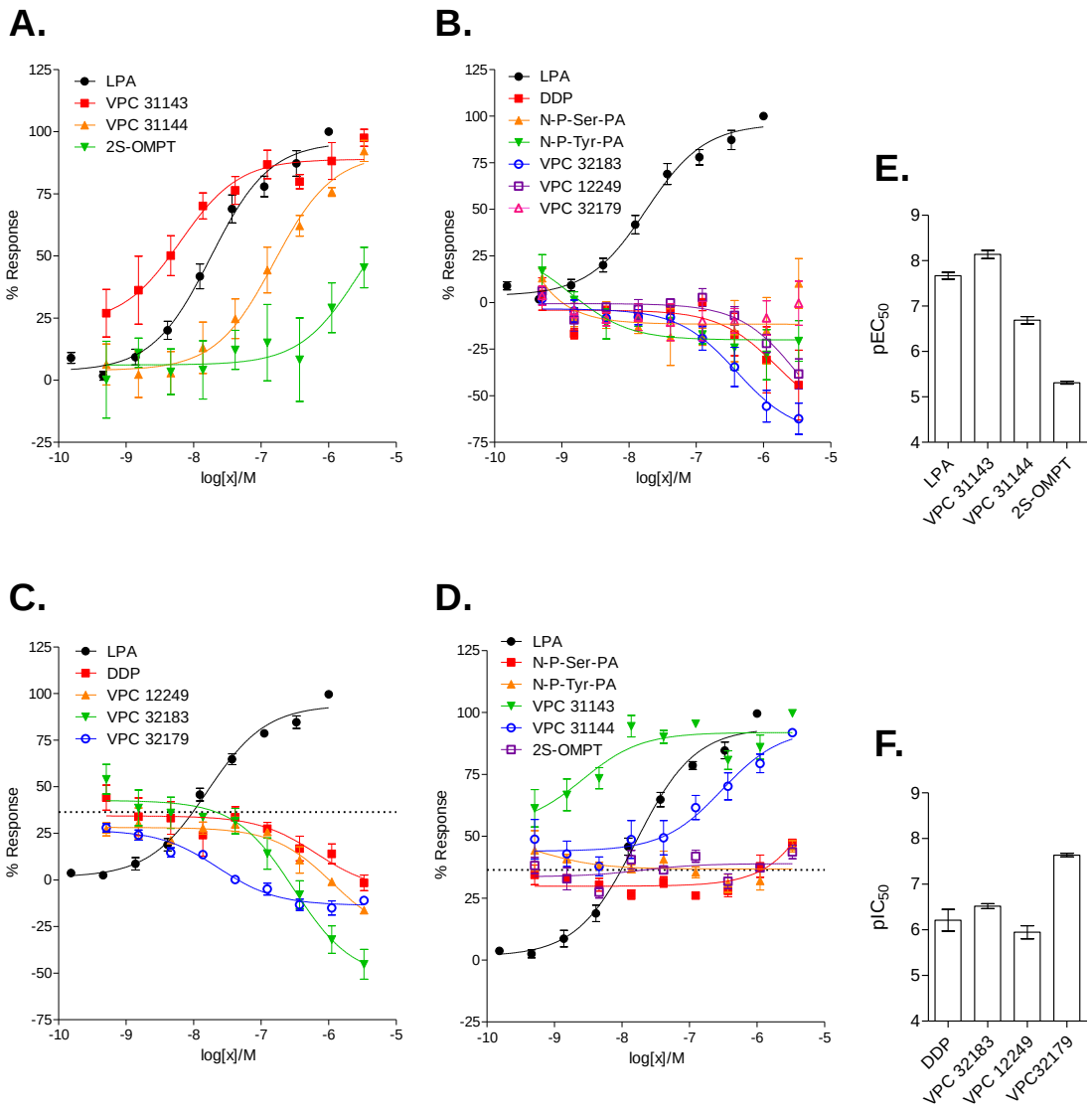
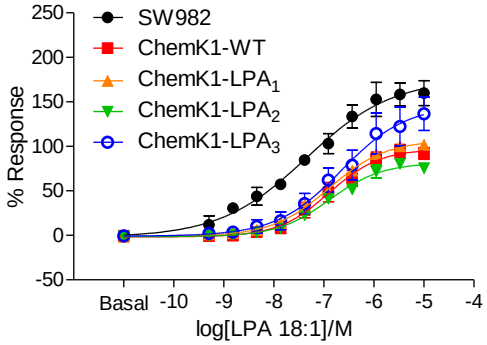


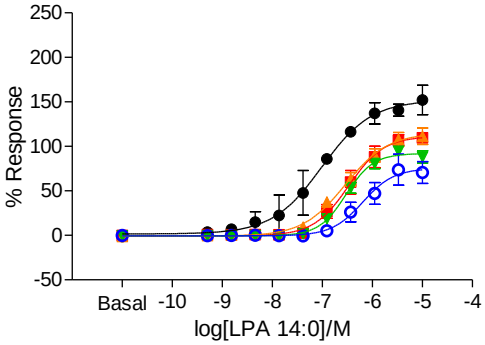
Figure 4.8

Agonist and antagonist effects of commercially-available lipids on [^{35}S]GTPγS 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.

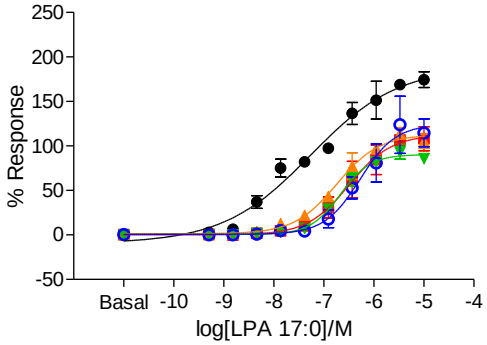
A. LPA 18:1



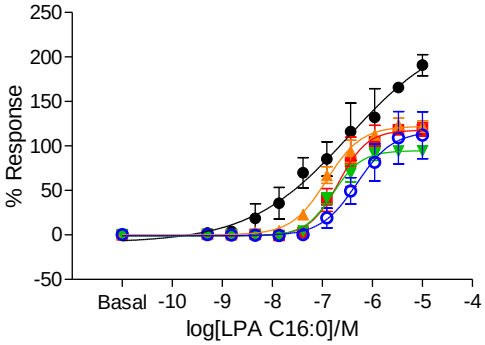
B. LPA 14:0



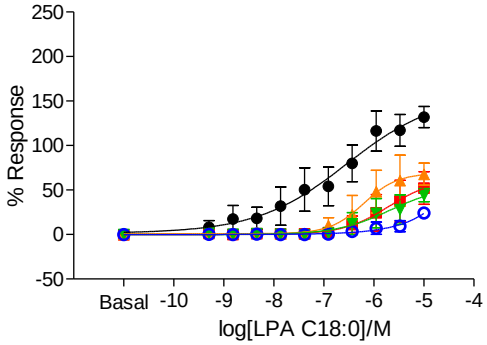
C. LPA 17:0



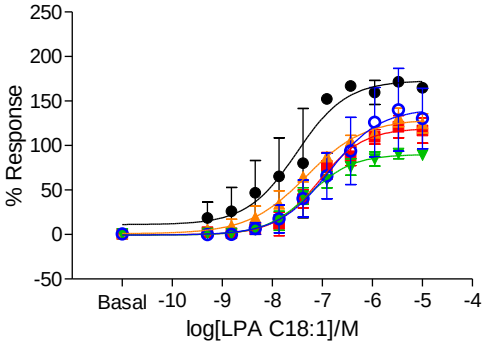
D. LPA C16:0



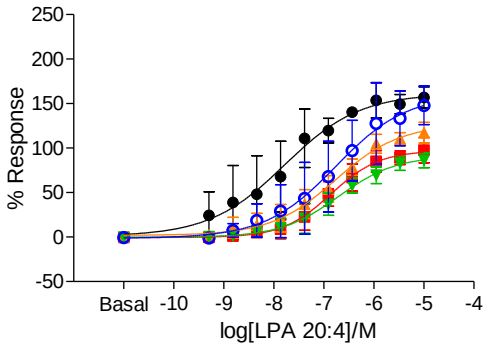
E. LPA C18:0



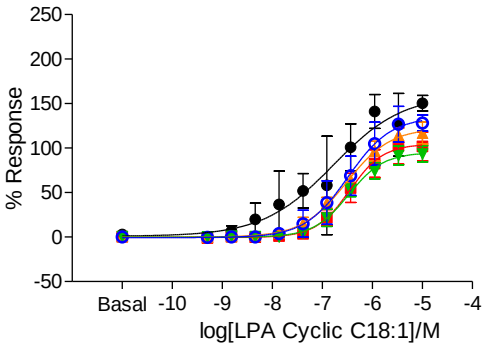
F. LPA C18:1



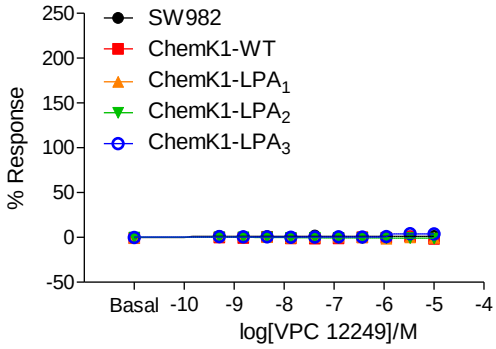
G. LPA 20:4



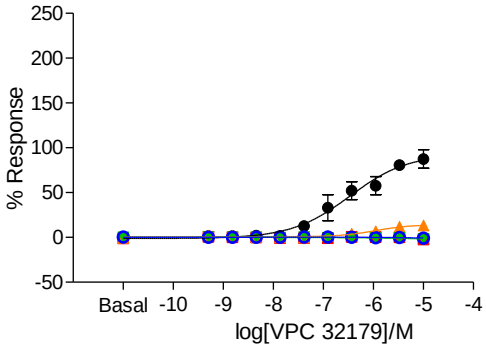
H. LPA Cyclic C18:1



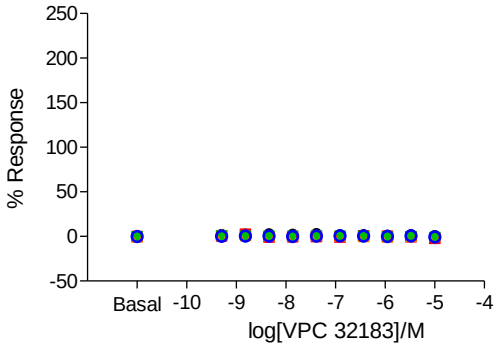
I. VPC 12249



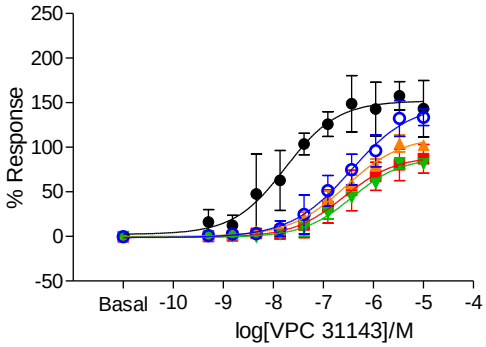
J. VPC 32179



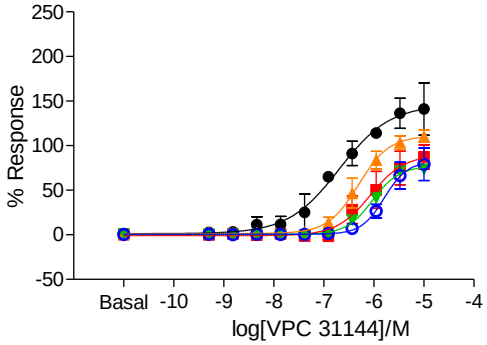
K. VPC 32183



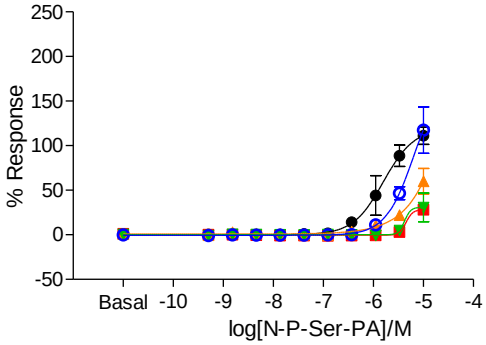
L. VPC 31143



M. VPC 31144



N. N-P-Ser-PA



O. N-P-Tyr-PA

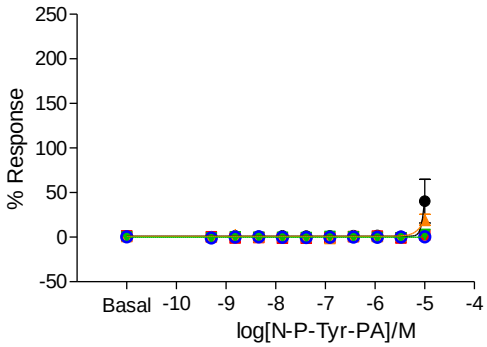


Figure 4.9

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-LPA_1 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.

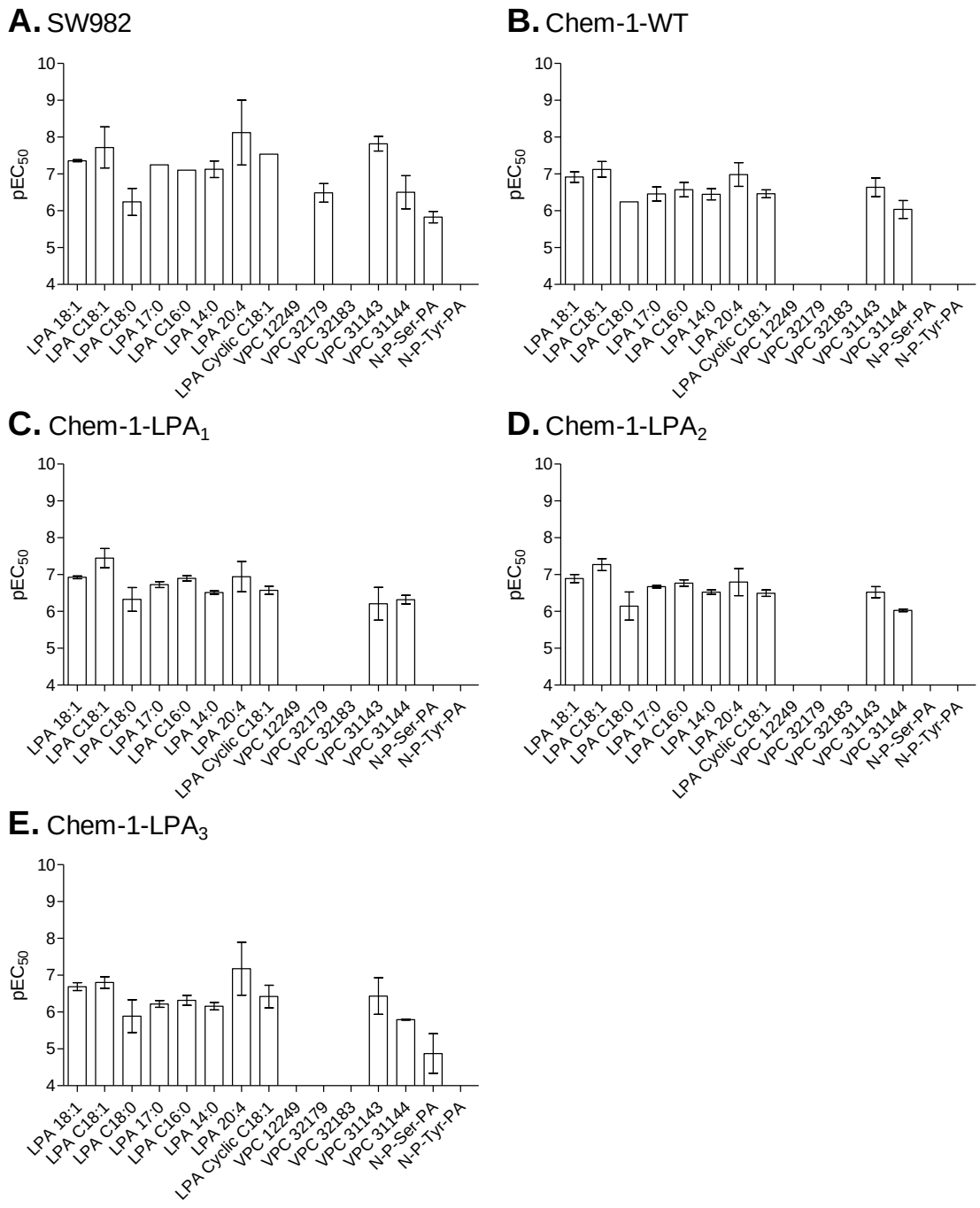


Figure 4.10

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, $p \leq 0.05$) 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]GTPγS 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]GTPγS 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 r*Lpar4-6*. All primers successfully detected their intended target only, and none of the other targets tested, with the exception of h*LPAR2* primers, which also detect r*Lpar2* at a low efficiency (Figure 4.12).

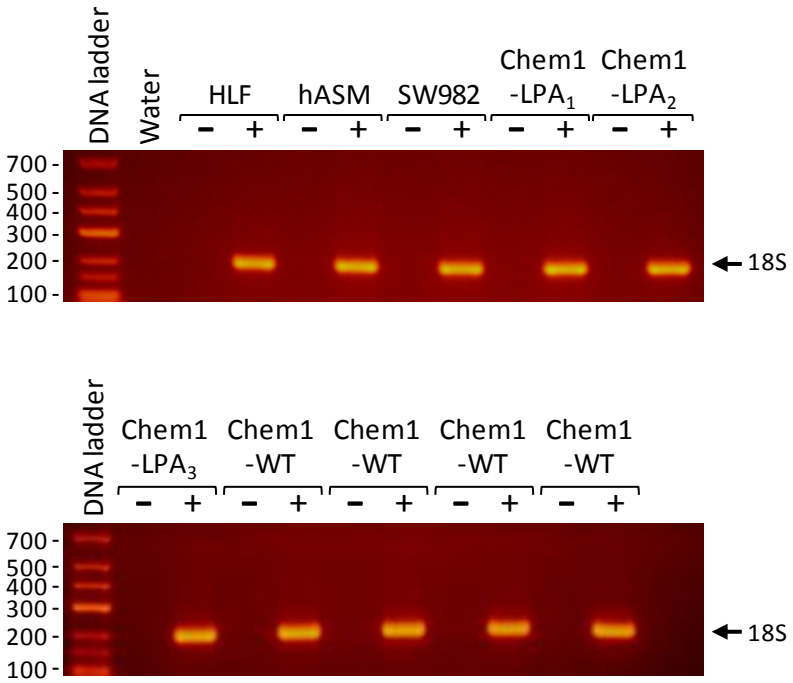


Figure 4.11

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.

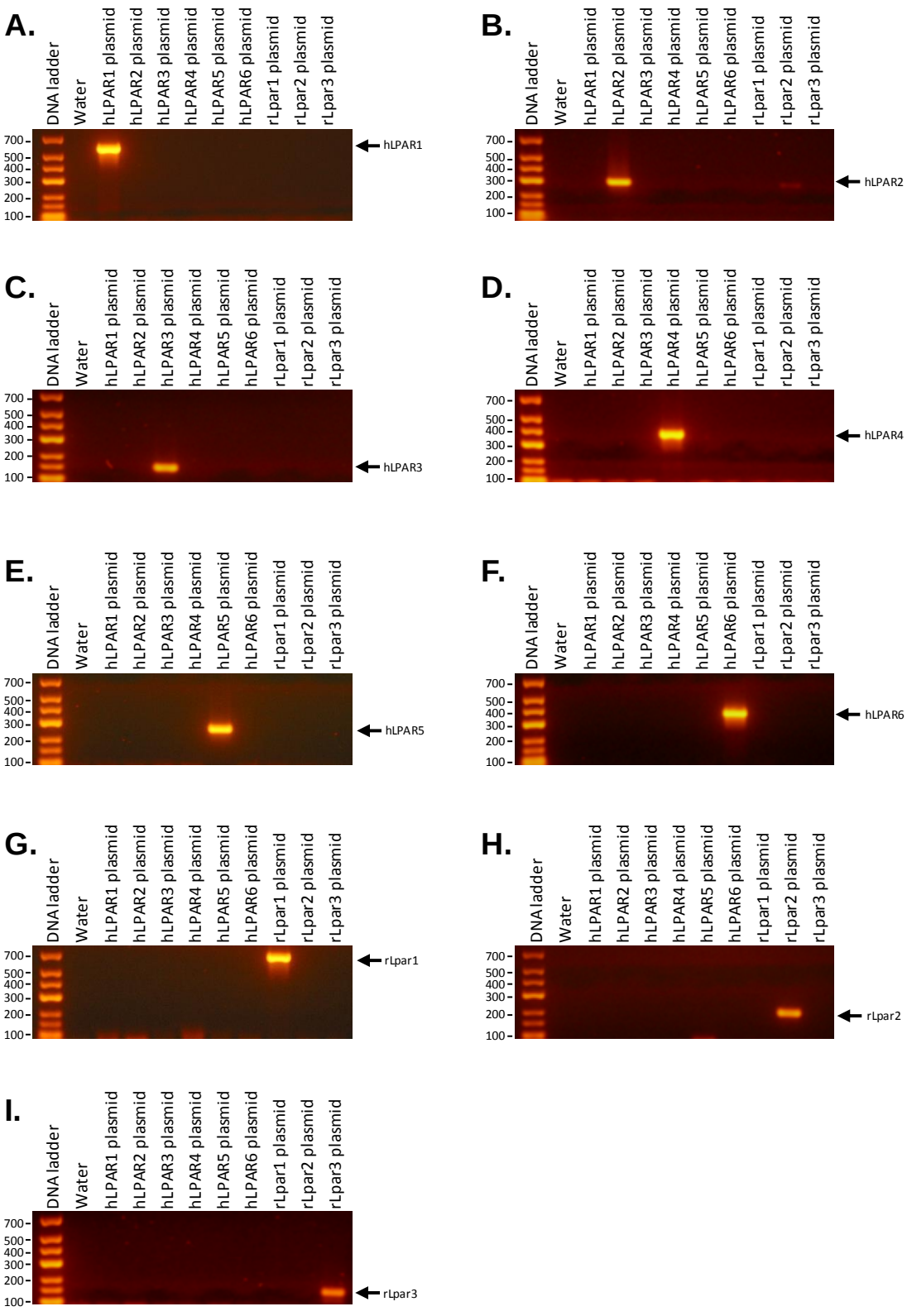

A.
DNA ladder
Water
hLPAR1 plasmid
hLPAR2 plasmid
hLPAR3 plasmid
hLPAR4 plasmid
hLPAR5 plasmid
hLPAR6 plasmid
rLpar1 plasmid
rLpar2 plasmid
rLpar3 plasmid
700
500
400
300
200
100
hLPAR1
B.
hLPAR2
C.
hLPAR3
D.
hLPAR4
E.
hLPAR5
F.
hLPAR6
G.
rLpar1
H.
rLpar2
I.
rLpar3

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 h*LPAR1-6*, respectively, and 648, 175 and 135 bp for r*Lpar1-3*, 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; r*Lpar-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 LPA_1 and LPA_3 cell-lines expressed the appropriate human LPA receptor, but no h*LPAR2* was detected in the LPA_2 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).

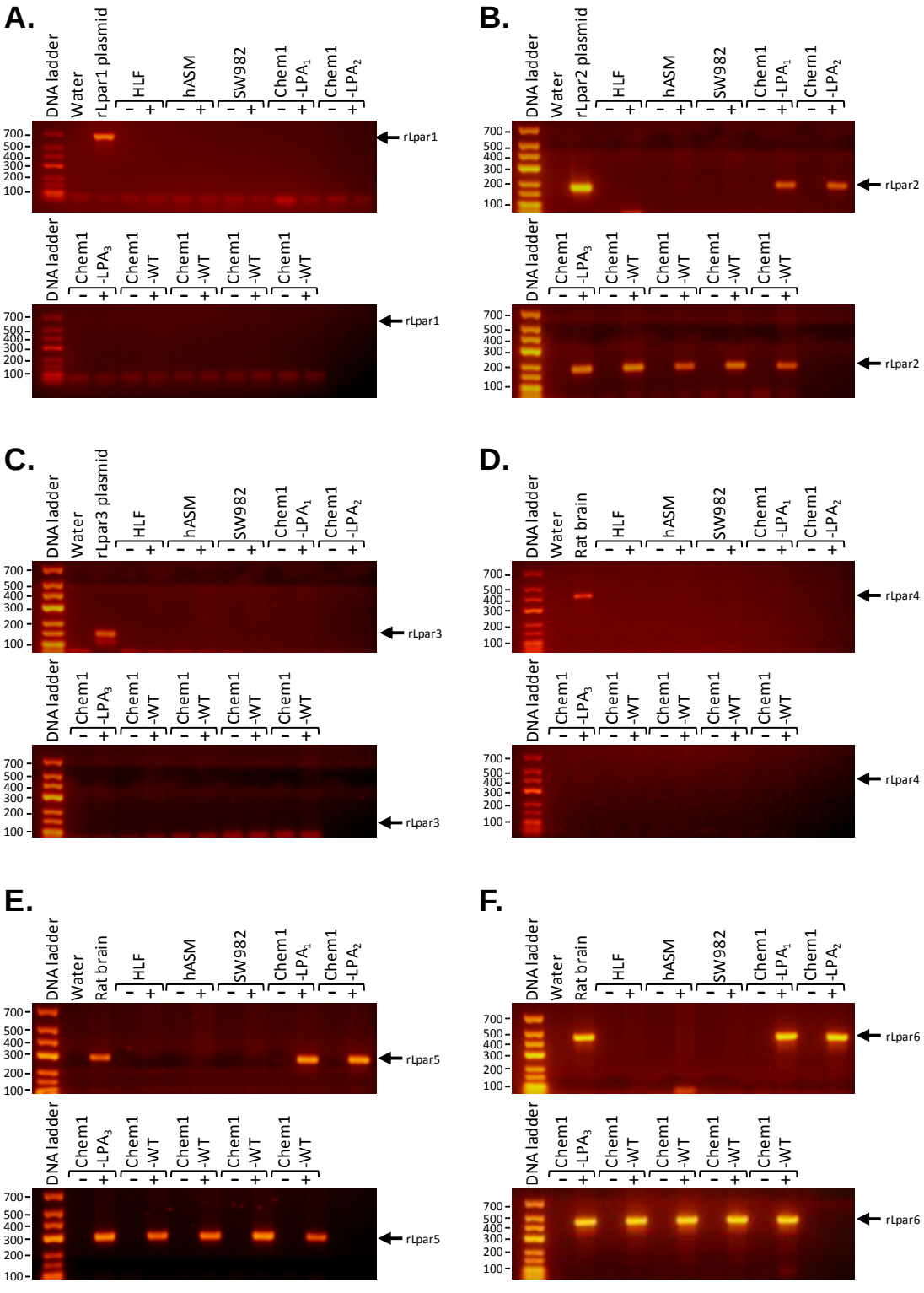

A.
DNA ladder
Water
rLpar1 plasmid
HLF
hASM
SW982
Chem1 -LPA1
Chem1 -LPA2
Chem1 -LPA3
Chem1 -WT
rLpar1
B.
rLpar2 plasmid
rLpar2
C.
rLpar3 plasmid
rLpar3
D.
Rat brain
rLpar4
E.
rLpar5
F.
rLpar6
700
500
400
300
200
100

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 r*Lpar* gene were used as PCR template for a positive control for r*Lpar1-3*. For r*Lpar4-6*, 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 r*Lpar1-6*, respectively.

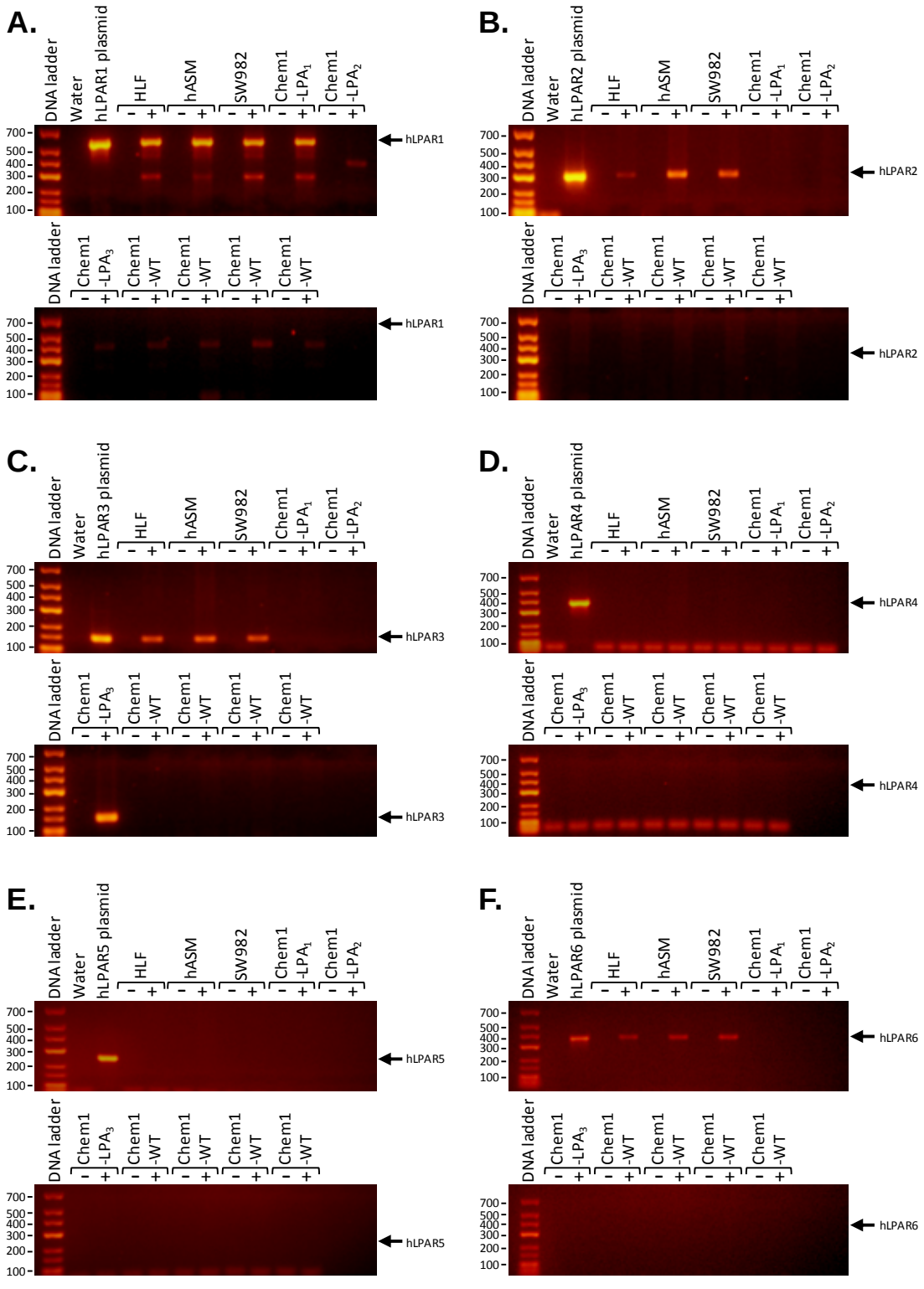

A.
DNA ladder
Water
hLPAR1 plasmid
HLF
hASM
SW982
Chem1 -LPA1
Chem1 -LPA2
Chem1 -LPA3
Chem1 -WT
hLPAR1
B.
hLPAR2 plasmid
hLPAR2
C.
hLPAR3 plasmid
hLPAR3
D.
hLPAR4 plasmid
hLPAR4
E.
hLPAR5 plasmid
hLPAR5
F.
hLPAR6 plasmid
hLPAR6
700
500
400
300
200
100

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 h*LPAR1-6*, respectively.

	Human LPA Receptor						Rat LPA receptor					
	1	2	3	4	5	6	1	2	3	4	5	6
HLF	+	+	+	-	-	+	-	-	-	-	-	-
hASM	+	+	+	-	-	+	-	-	-	-	-	-
SW982	+	+	+	-	-	+	-	-	-	-	-	-
Chem1-LPA_1	+	-	-	-	-	-	-	+	-	-	+	+
Chem1-LPA_2	-	-	-	-	-	-	-	+	-	-	+	+
Chem1-LPA_3	-	-	+	-	-	-	-	+	-	-	+	+
Chem1-WT	-	-	-	-	-	-	-	+	-	-	+	+

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}S]GTPγS binding, specific [^{3}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}S]GTPγS 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}S]GTPγS 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}S]GTPγS and 20 µg/well membrane protein to achieve adequate levels of [^{35}S]GTPγS 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}S]GTPγS 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}S]GTPγS 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 $G\alpha_{i/o}$ 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 Gα subtypes, the basal [^{35}S]GTPγS binding attributable to $G\alpha_{i/o}$-[^{35}S]GTPγS complexes may mask the contribution of other Gα subtypes to the total [^{35}S]GTPγS binding (Milligan, 2003). To assess the contribution of $G\alpha_{i/o}$-[^{35}S]GTPγS 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}S]GTPγS binding in RH7777-LPA_1 cell membranes to levels observed in membranes prepared from RH7777 wild-type cells, indicating that other Gα subtypes are likely to make at most, a minor contribution to the total receptor-activated [^{35}S]GTPγS binding. However, RH7777 cells are known to express a range of Gα subtypes and LPA_1 is proposed to couple to members of the $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ 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 $G\alpha_{i/o}$-[^{35}S]GTPγS complexes.

It is interesting to note that expression of the LPA_1 receptor causes a marked increase in the basal level of [^{35}S]GTPγS 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 LPA_1 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-LPA_1 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 [^{35}S]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 [^{35}S]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 [^{35}S]GTPγS binding assay was used to compare the effects of LPAs with varying chain lengths, degrees of saturation and ester/ether linker in RH7777-LPA_1-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 [^{35}S]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}S]GTPγS 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}S]GTPγS 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}S]GTPγS 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}S]GTPγS 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}S]GTPγS 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 Ca^{2+} 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-LPA_1 membranes. This suggests that inhibition of the LPA-induced Cl^- current and Ca^{2+} responses induced by these compounds are not likely to be mediated by G proteins coupled to LPA_1.

Following investigation of the action of these lipids at LPA_1 containing membranes using a [^{35}S]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 LPA_1, LPA_2 or LPA_3 receptor subtype purchased from Millipore. Chem-1 cells over-express $G\alpha_{15}$, allowing most GPCR subtypes to produce robust Ca^{2+} responses upon agonist challenge, enabling testing of the lipids at these three LPA receptor subtypes using a high throughput Ca^{2+} assay.

It was thought that it would be more efficient to develop the Chem-1/Ca^{2+} assay system to perform the lipid screen than to develop new cell-lines to enable continuation of the work using the [^{35}S]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 Ca^{2+} 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 h*LPAR1-6* or r*Lpar1-3* showed that the primers could detect, and were specific for, their intended targets. It is worth noting that primers for h*LPAR2* also detect a faint band when used to probe the r*Lpar2* 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 r*Lpar4-6* were unavailable for these experiments so the specificity of the r*Lpar4-6* 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 r*Lpar4-6* 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 they 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 r*Lpar-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 h*LPAR-1* and *-3* sequences are detected in the appropriate transfected Chem-1 cell-lines, there was no detection of h*LPAR2* 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 h*LPAR-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 LPA_1 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 LPA_1 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 LPA_1 did not display the LPA-induced chemotactic response observed in fibroblasts from wild-type mice, suggesting that LPA_1 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 [^{35}S]GTPγS binding assay was developed and effectively used to investigate lipid activities at LPA_1 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 Ca^{2+}

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. Unfortunately, the LPA-responsiveness of Chem-1wild-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]GTPγS 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]GTPγS 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 LPA_1 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 LPA_1, LPA_2, and LPA_3 are reported to reduce cellular cAMP concentration by coupling to $G\alpha_i$ proteins and inhibiting adenylyl cyclase (AC) activity (Ishii *et al.*, 2000), while stimulation of LPA_4 and LPA_5 leads to an increase in cAMP levels by coupling to $G\alpha_s$ 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 β_2-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 $E_{1,}$ which stimulate cAMP production through direct AC activation, β_2-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 β_2-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 $G\alpha_i$ 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, β_2-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 5.1**A**). 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, $p \leq 0.01$, 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 5.1**B**). 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

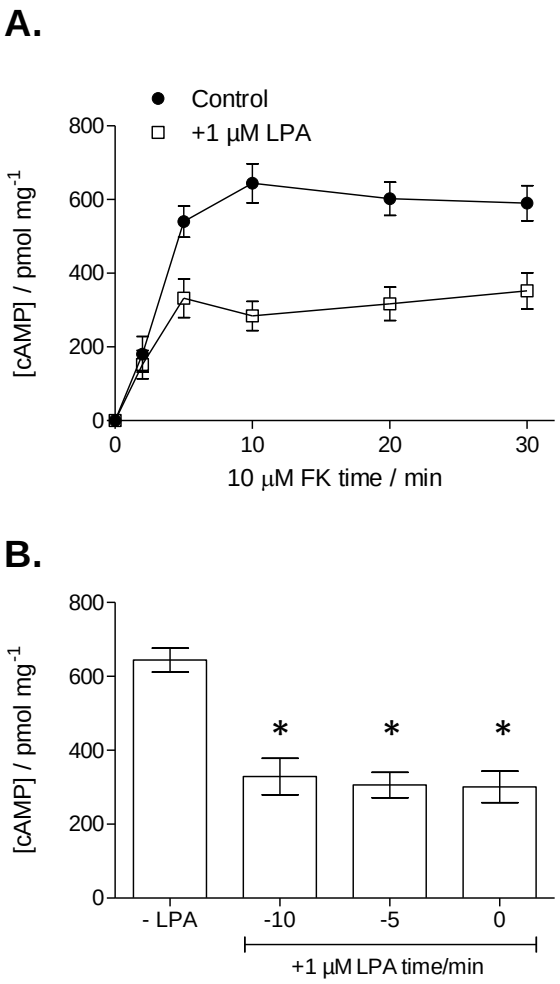


Figure 5.1

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.

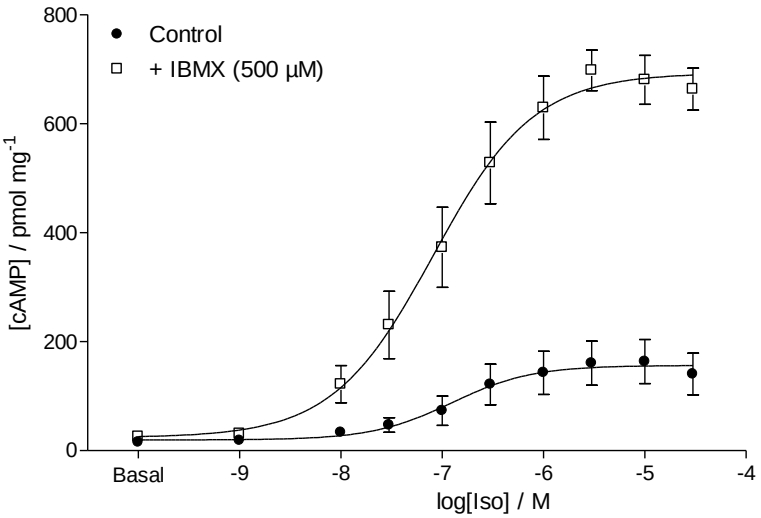


Figure 5.2

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 pEC_{50} 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 $G\alpha_s$ 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 $LPA_{1/3}$-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 $G\alpha_i$ 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 5.5**A**).

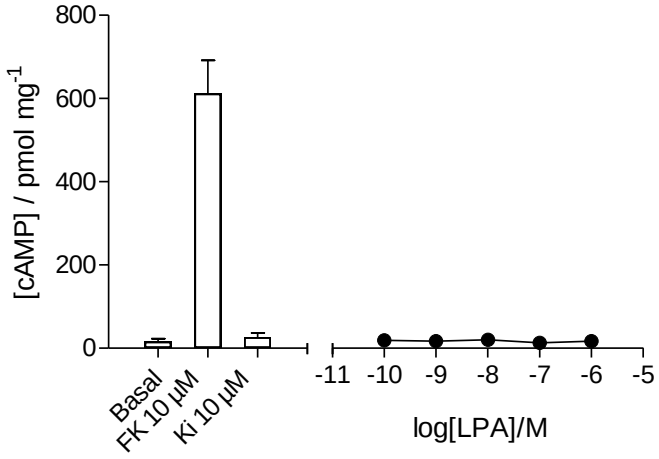


Figure 5.3

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.

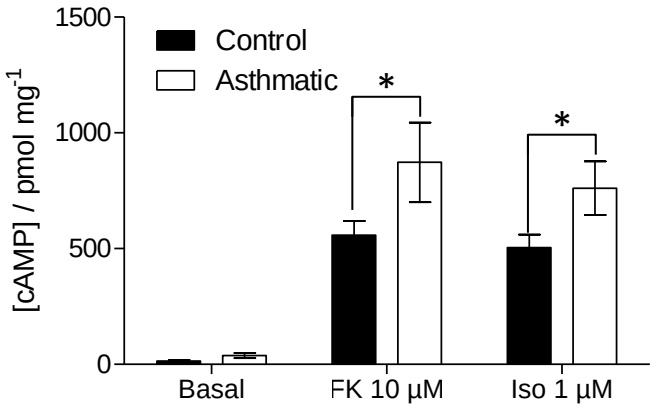


Figure 5.4

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, $p \leq 0.05$. 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.

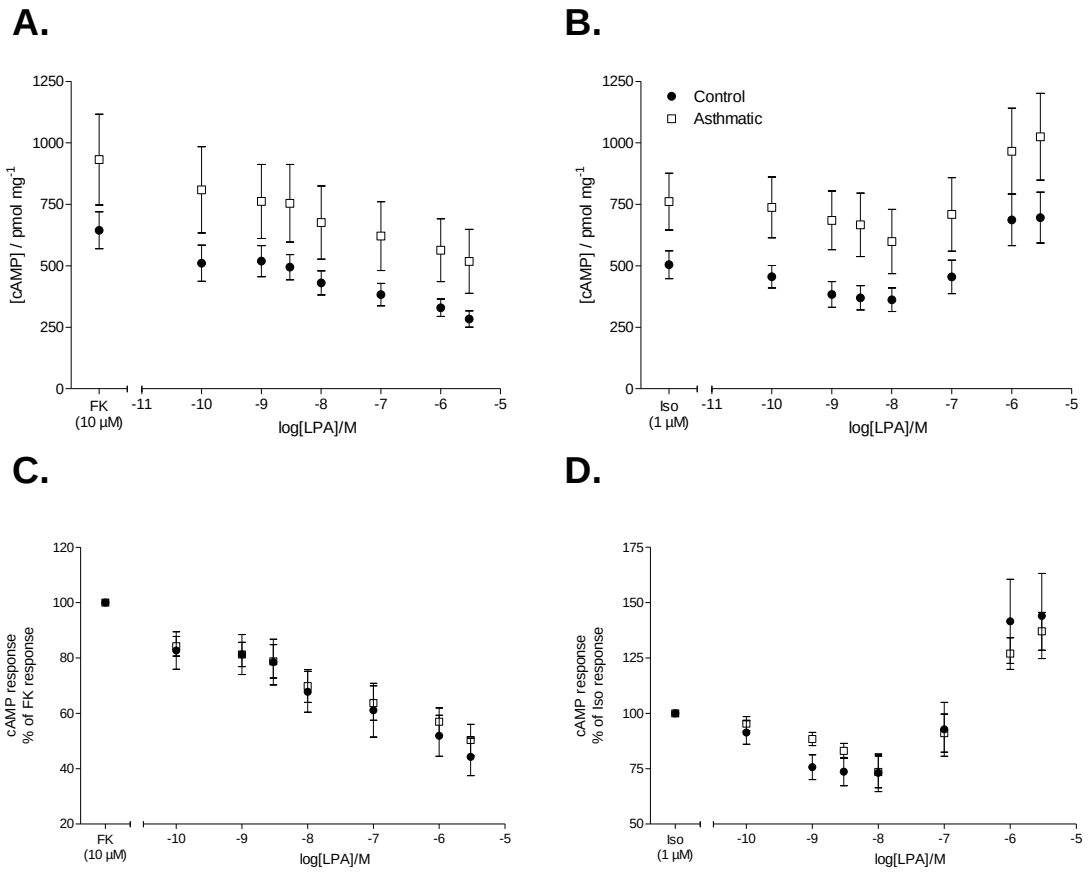


Figure 5.5

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.

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 5.5**B**). Cells derived from control donors showed basal cAMP levels of 14.7 ± 4.5 pmol mg^{-1}. Isoprenaline (1 µM) induced a response of 504.4 ± 56.4 pmol mg^{-1} which was reduced by 28.2 % to 362.0 ± 47.8 pmol mg^{-1} at 10 nM LPA, but increased to 686.9 ± 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 ± 11.5 pmol mg^{-1}. Isoprenaline (1 µM) induced a response of 761.1 ± 115.4 which was reduced by 21.3 % to 598.7 ± 130.6 pmol mg^{-1} at 10 nM LPA, which then increased to 966.0 ± 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 5.5**C** 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).

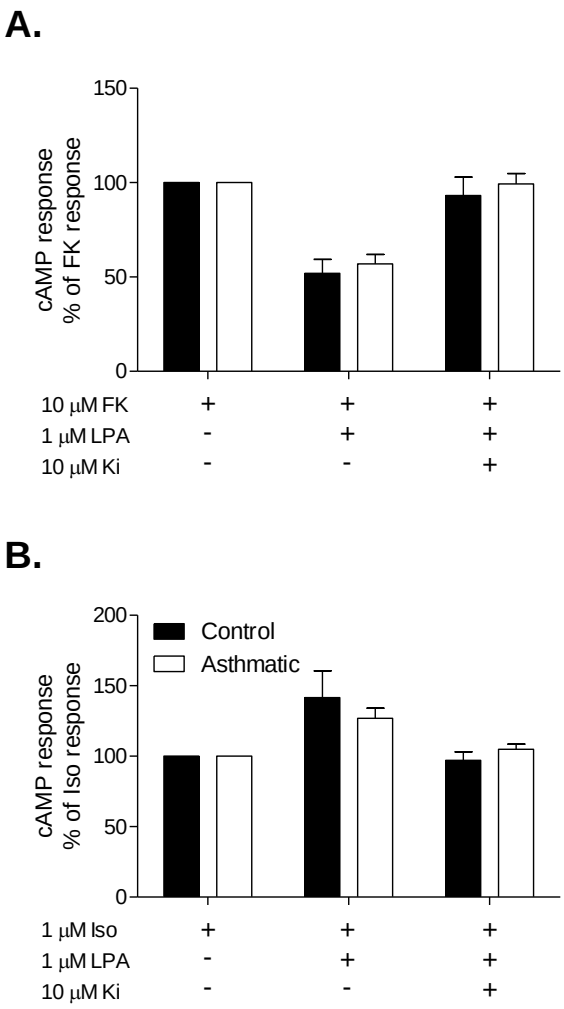


Figure 5.6

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.

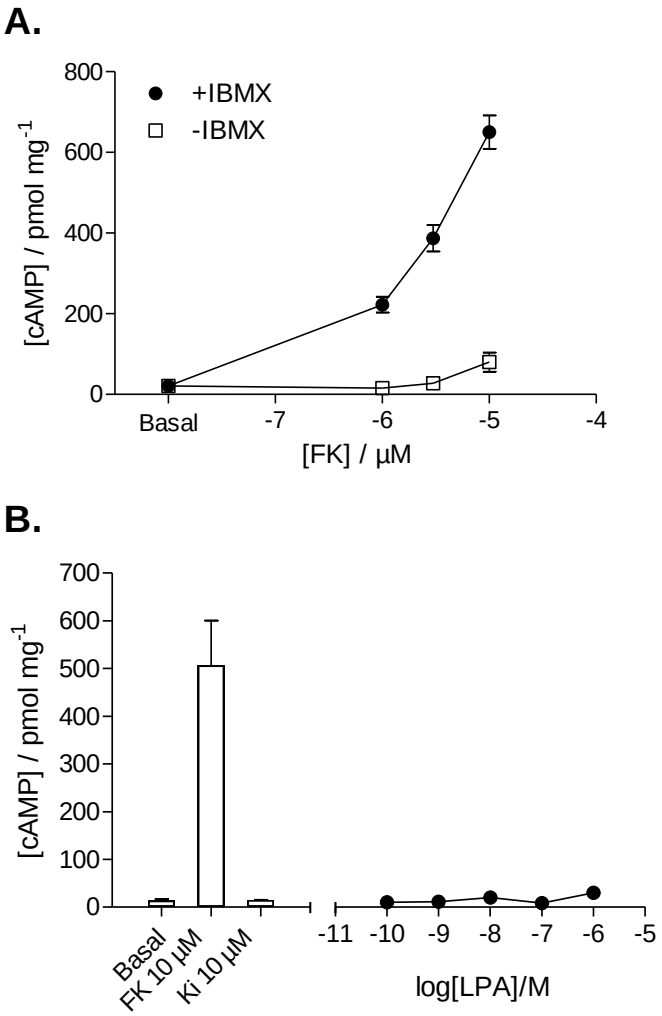


Figure 5.7

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.

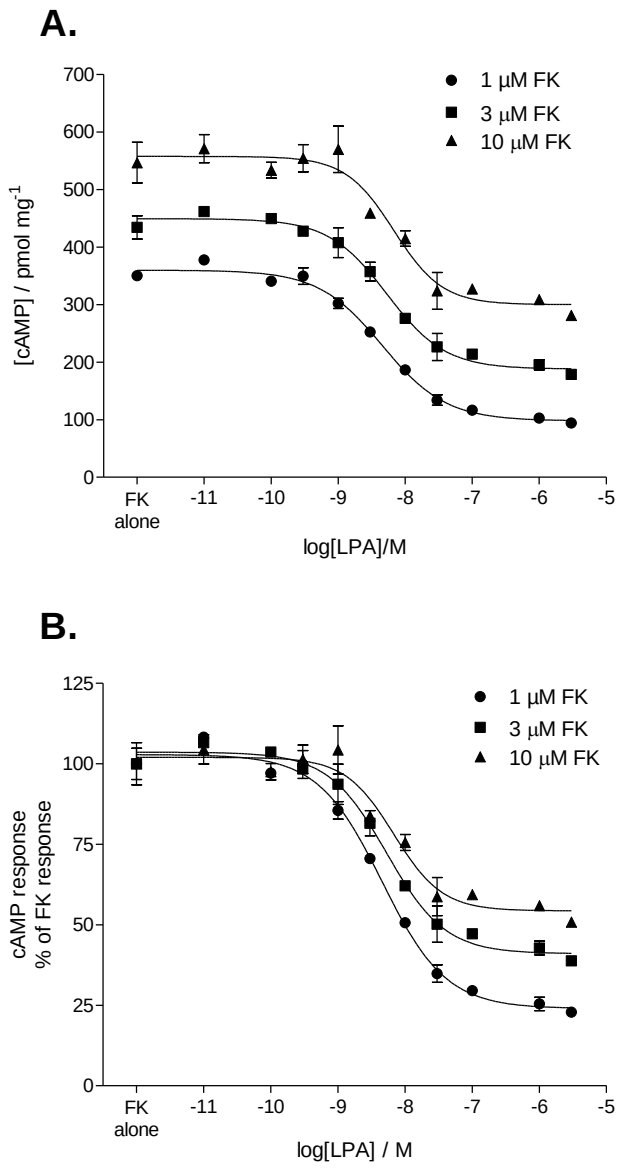


Figure 5.8

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 5.8**A**), 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 5.8**B**). The observed pIC_{50}s 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 $LPA_{1/3}$-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 $G\alpha_i$-mediated aspect of the modulation (Figure 5.11**A**).

Removal of Ca^{2+} from the incubation medium and inclusion of the Ca^{2+} chelator EGTA (100 µM), had only a small effect on the LPA-mediated modulation of the isoprenaline cAMP response (Figure 5.11**B**): At 1-10 nM, LPA the extent of the inhibitory

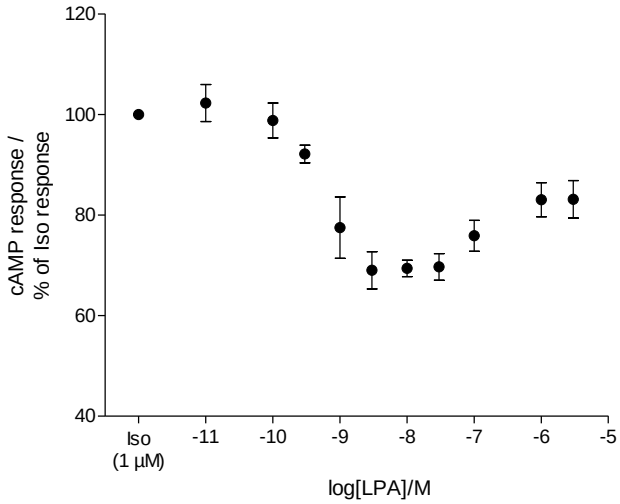


Figure 5.9

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.

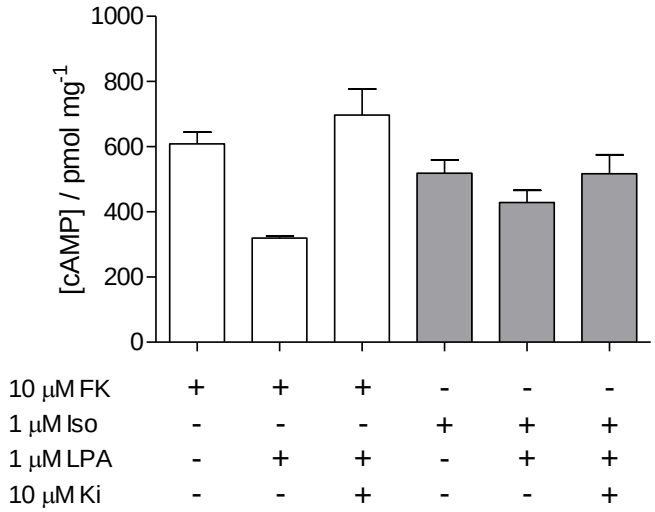


Figure 5.10

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.

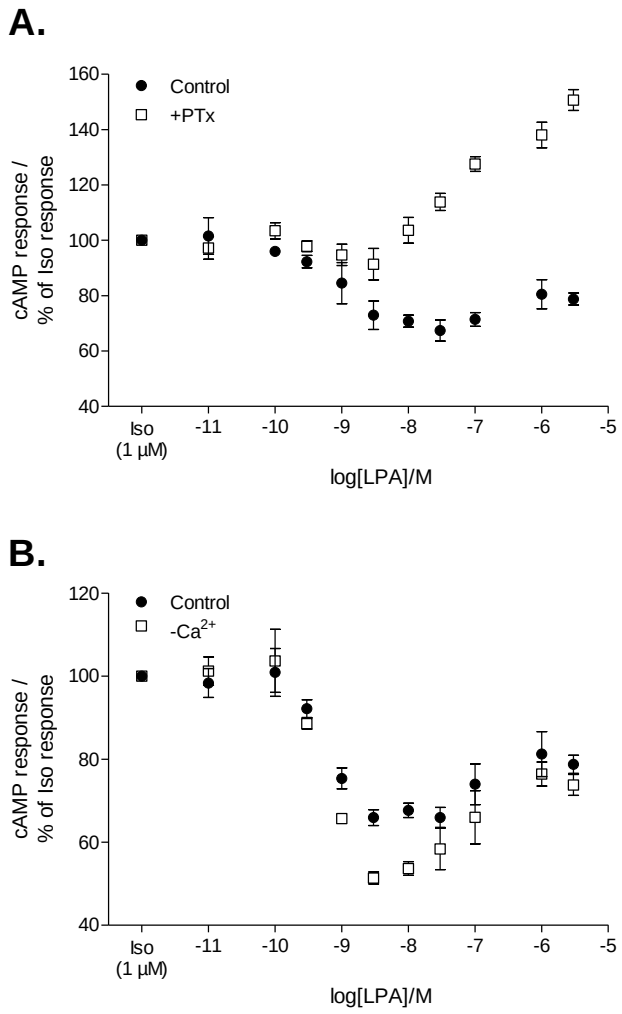


Figure 5.11

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 5.11**B**), 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 5.13**A**). 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 5.13**B**). 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

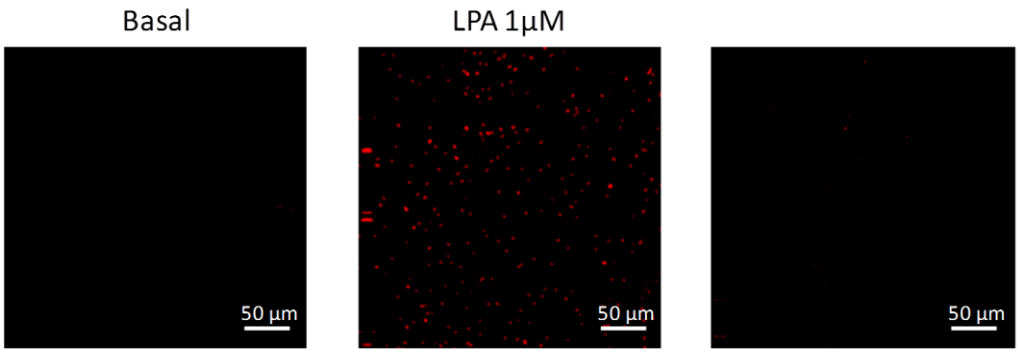


Figure 5.12

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.

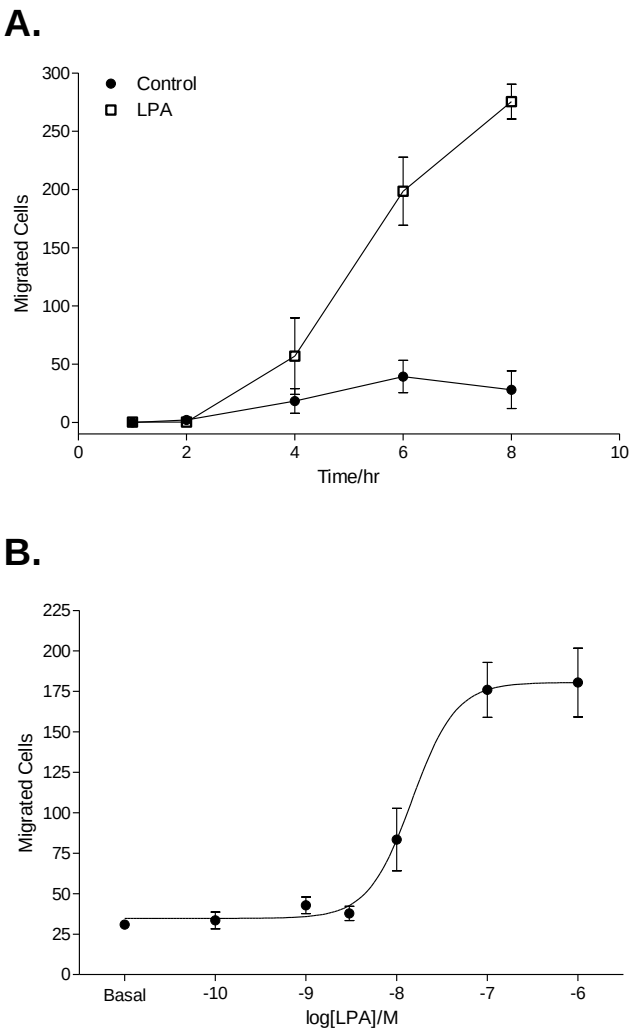


Figure 5.13

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.

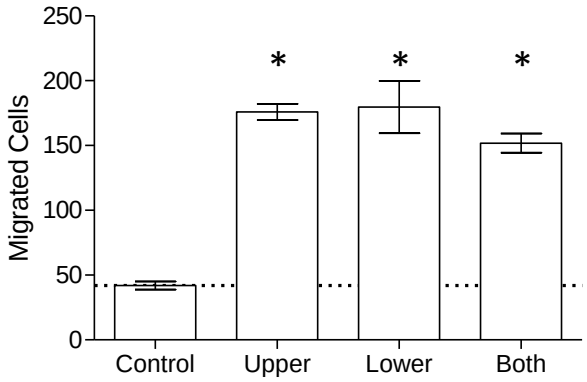


Figure 5.14

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 5.15**A**). 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 $LPA_{1/3}$-selective antagonist, Ki16425, strongly suggesting that the observed response is LPA-receptor mediated (Figure 5.15**A**). 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 $G\alpha_i$ dependency(Figure 5.15**B**). 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

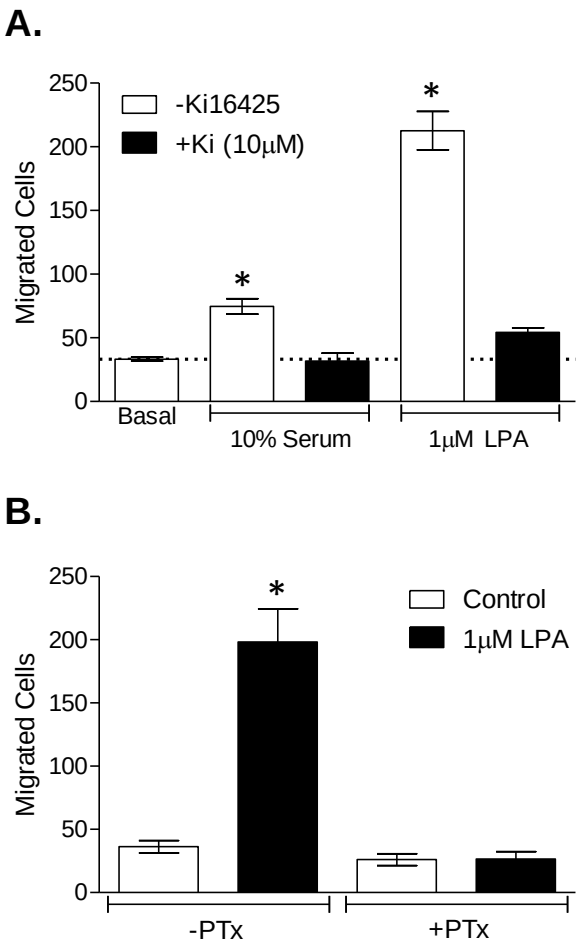


Figure 5.15

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.

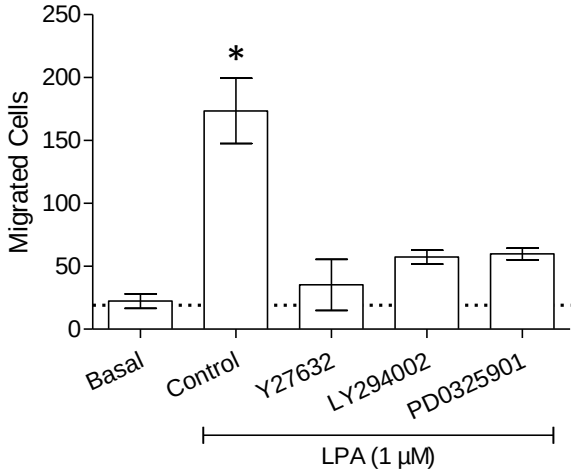


Figure 5.16

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 ± 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.

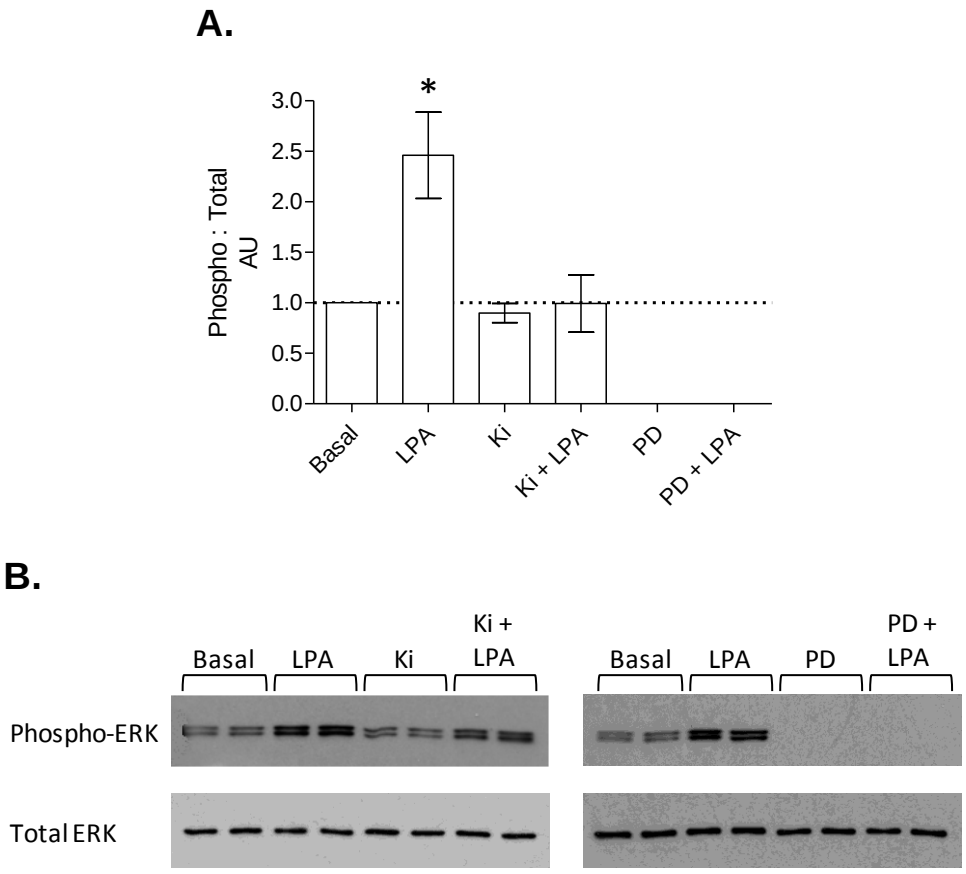


Figure 5.17

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 5.1**A**). Still, LPA pre-treatment was able to significantly reduce the observed response (Figure 5.1**A**). The time-course of LPA treatment also appeared to be quite stable (Figure 5.1**B**) 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 $G\alpha_s$ 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 $G\alpha_s$ 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 β_2-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 β_2-adrenoceptor activity and that prolonged exposure to β_2-adrenoceptor agonists can lead to desensitisation of β_2-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 β_2-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 β_2-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 5.5**A** 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 5.5**C** and **D**). LPA inhibited the forskolin-induced cAMP response in a concentration-dependent fashion (Figure 5.5**A** and **C**), as would be predicted by the existing literature reporting $G\alpha_i$ 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 $G\alpha_i$ 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 5.5**B** 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 5.7**A**) and LPA elicited no cAMP response itself (Figure 5.7**B**). 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 $LPA_{1/3}$-selective antagonist, Ki16425, for 10 min prior to LPA addition, neutralised the effects of LPA on forskolin- or isoprenaline- induced cAMP responses (Figure 5.10), indicating that the observed effects are $LPA_{1/3}$-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 $G\alpha_i$-mediated, and secondly, that this $G\alpha_i$-mediated effect suppresses the potentiating effects of LPA treatment, which are not mediated by $G\alpha_i$ 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 $G\alpha_s$ at AC2 is known to be potentiated by $G\beta\gamma$ subunits (Tang and Gilman, 1991; Federman *et al.*, 1992) so it is possible that LPA exerts its potentiating effect on isoprenaline responses by contributing $G\beta\gamma$ 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 $G\alpha_s$ 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 $G\beta\gamma$ subunits, these must be derived from G-protein trimers containing a $G\alpha$ subunit other than $G\alpha_i$. Since many LPA receptors, including LPA_1 and LPA_3, 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 $G\alpha_i$ (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 $G\alpha_i$ 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 5.11**B**). 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 5.15**A**). 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 $G\alpha_i$ (Figure 5.15**B**). 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 $G\alpha_i$ in mediating cell migration in response to activation of a range of other GPCRs, including the PAF receptor, CXC chemokine receptors and $S1P_1$ (Brown *et al.*, 2006; Thompson *et al.*, 2007; Dutt *et al.*, 1998; Yoon *et al.*, 2008). Despite the implication of $G\alpha_i$, 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 β_2-adrenoceptor expression with β_2-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.

CHAPTER 6. Discussion

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}S]GTPγS 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}S]GTPγS binding, which may indicate constitutive activity of LPA_1 or a significant concentration of LPA endogenous to the membranes generated. All [^{35}S]GTPγS 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 (2*S*)-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 $hLPA_1$ 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 $G\alpha_{15}$ to facilitate coupling to a Ca^{2+} 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/$G\alpha_{15}$ cells for investigation of LPA receptor subtype-specific effects. Observed LPA and lipid responses were similar to those seen for [^{35}S]GTPγS binding in RH7777-LPA_1 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 Ca^{2+} 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 β_2-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 β_2-adrenoceptor-mediated cAMP responses. The effects of treatment with 1 μM LPA on AC- and β_2-

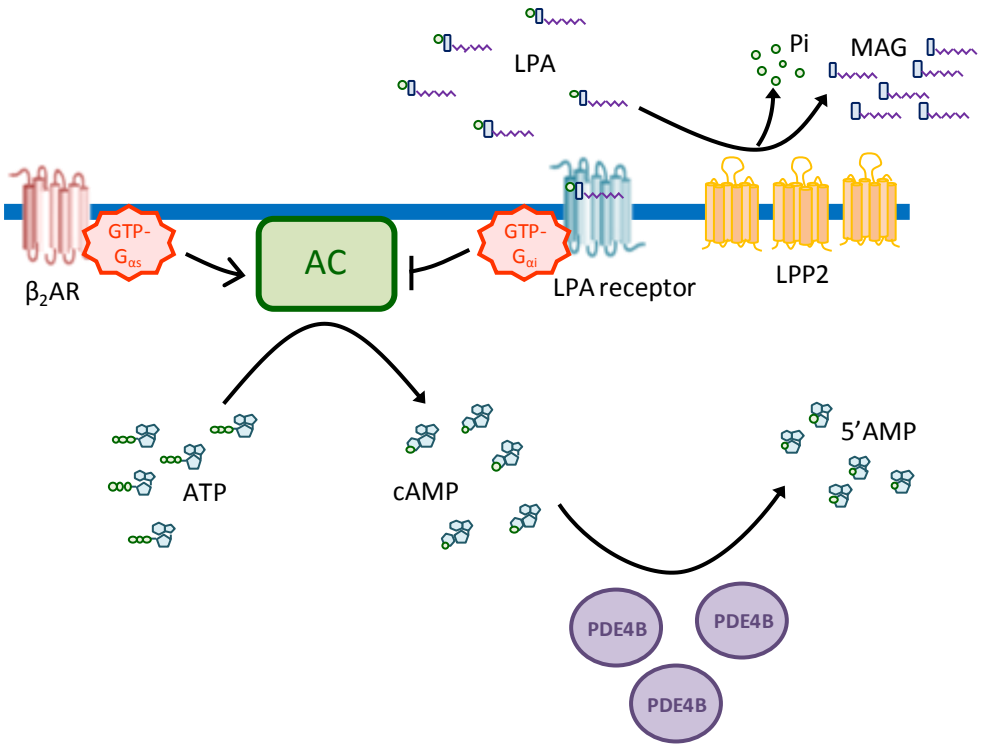
adrenoceptor-mediated cAMP responses were completely prevented by pre-treatment with the $LPA_{1/3}$-selective antagonist Ki16425. Though LPA was shown to modulate cAMP responses in hASM cells, no disease state-dependent effects of LPA were apparent.

Though hASM cells were the primary focus of this work, SW982 cells were also used as a model of LPA_1-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 β_2-adrenoceptor-mediated cAMP responses were recapitulated in this model cell line. Further investigation revealed that the inhibitory phase of LPA action on β_2-adrenoceptor-mediated cAMP response is $G\alpha_i$-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 $LPA_{1/3}$-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

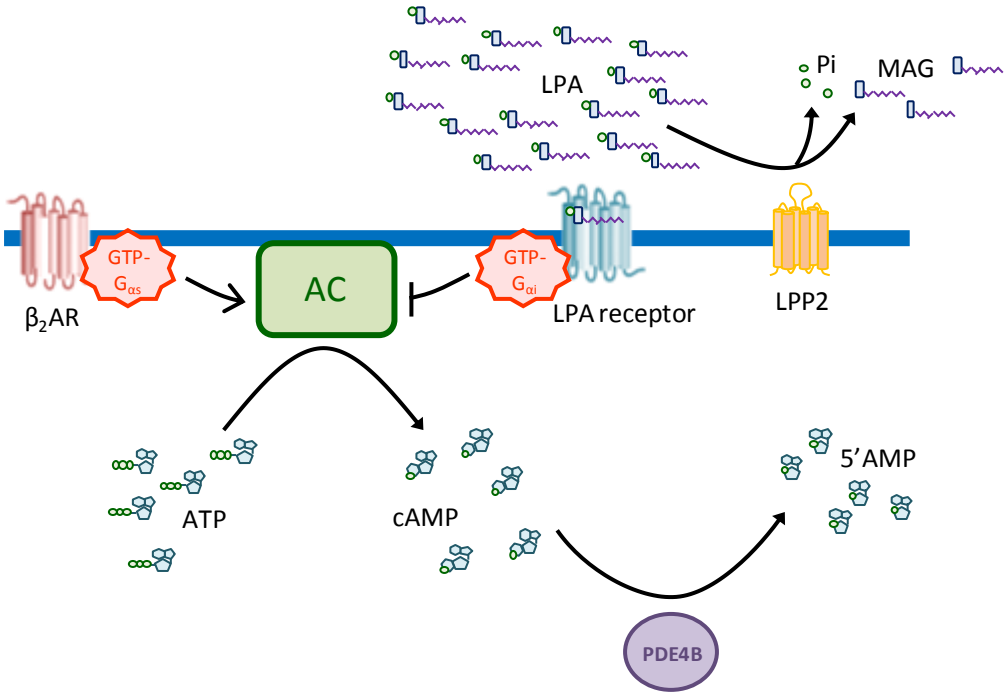


Figure 6.1

Summary of the work presented in this thesis. In hASM, LPA modulates cAMP production, whether mediated by β_2-adrenoceptors (β_2AR) 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 LPA_1 or LPA_3 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 andFigure 5.5). Work in the SW982 model cell line indicates that cAMP modulation is mediated, in part, by $G_{\alpha i}$ 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 *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. *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 *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 $G\alpha_s$ 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 *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}S]GTPγS 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.

Bibliography

Aaltonen N, Palomaki VA, Lecklin A and Laitinen JT (2008) Neuroanatomical mapping of juvenile rat brain regions with prominent basal signal in [(35)S]GTPgammaS autoradiography. *J Chem Neuroanat* **35:**233-241.

Adolfsson PI, Ahlstrand C, Varenhorst E and Svensson SP (2002) Lysophosphatidic acid stimulates proliferation of cultured smooth muscle cells from human BPH tissue: sildenafil and papaverin generate inhibition. *Prostate* **51:**50-58.

Alexander SP, Mathie A and Peters JA (2011) Guide to Receptors and Channels (GRAC), 5th edition. *Br J Pharmacol* **164 Suppl 1:**S1-324.

Ali G, Chishti MS, Raza SI, John P and Ahmad W (2007) A mutation in the lipase H (LIPH) gene underlie autosomal recessive hypotrichosis. *Hum Genet* **121:**319-325.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y and Kaibuchi K (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* **271:**20246-20249.

Amisten S, Braun OO, Bengtsson A and Erlinge D (2008) Gene expression profiling for the identification of G-protein coupled receptors in human platelets. *Thromb Res* **122:**47-57.

Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, Kane SA, Peters SP, Penn RB, Spiegel S and Panettieri RA,Jr (2001) Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J* **15:**1212-1214.

Ammit AJ, Hoffman RK, Amrani Y, Lazaar AL, Hay DW, Torphy TJ, Penn RB and Panettieri RA,Jr (2000) Tumor necrosis factor-alpha-induced secretion of RANTES and interleukin-6 from human airway smooth-muscle cells. Modulation by cyclic adenosine monophosphate. *Am J Respir Cell Mol Biol* **23:**794-802.

An S, Bleu T, Hallmark OG and Goetzl EJ (1998) Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J Biol Chem* **273:**7906-7910.

An S, Dickens MA, Bleu T, Hallmark OG and Goetzl EJ (1997) Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. *Biochem Biophys Res Commun* **231:**619-622.

Anliker B and Chun J (2004) Cell surface receptors in lysophospholipid signaling. *Semin Cell Dev Biol* **15:**457-465.

Aoki J, Inoue A and Okudaira S (2008) Two pathways for lysophosphatidic acid production. *Biochim Biophys Acta* **1781:**513-518.

Aoki J, Taira A, Takanezawa Y, Kishi Y, Hama K, Kishimoto T, Mizuno K, Saku K, Taguchi R and Arai H (2002) Serum lysophosphatidic acid is produced through diverse phospholipase pathways. *J Biol Chem* **277:**48737-48744.

AsthmaUK (2010) **Asthma facts and FAQs**. **2013:**1.

Attwood TK and Findlay JB (1994) Fingerprinting G-protein-coupled receptors. *Protein Eng* **7:**195-203.

Bafadhel M, McCormick M, Saha S, McKenna S, Shelley M, Hargadon B, Mistry V, Reid C, Parker D, Dodson P, Jenkins M, Lloyd A, Rugman P, Newbold P and Brightling CE (2012) Profiling of sputum inflammatory mediators in asthma and chronic obstructive pulmonary disease. *Respiration* **83:**36-44.

Bagga S, Price KS, Lin DA, Friend DS, Austen KF and Boyce JA (2004) Lysophosphatidic acid accelerates the development of human mast cells. *Blood* **104:**4080-4087.

Bai TR and Knight DA (2005) Structural changes in the airways in asthma: observations and consequences. *Clin Sci (Lond)* **108:**463-477.

Baker DL, Desiderio DM, Miller DD, Tolley B and Tigyi GJ (2001) Direct quantitative analysis of lysophosphatidic acid molecular species by stable isotope dilution electrospray ionization liquid chromatography-mass spectrometry. *Anal Biochem* **292:**287-295.

Baker DL, Umstot ES, Desiderio DM and Tigyi GJ (2000) Quantitative analysis of lysophosphatidic acid in human blood fractions. *Ann N Y Acad Sci* **905:**267-269.

Balazs L, Okolicany J, Ferrebee M, Tolley B and Tigyi G (2001) Topical application of the phospholipid growth factor lysophosphatidic acid promotes wound healing in vivo. *Am J Physiol Regul Integr Comp Physiol* **280:**R466-72.

Bandoh K, Aoki J, Hosono H, Kobayashi S, Kobayashi T, Murakami-Murofushi K, Tsujimoto M, Arai H and Inoue K (1999) Molecular cloning and characterization of a novel human G-protein-coupled receptor, EDG7, for lysophosphatidic acid. *J Biol Chem* **274:**27776-27785.

Bandoh K, Aoki J, Taira A, Tsujimoto M, Arai H and Inoue K (2000) Lysophosphatidic acid (LPA) receptors of the EDG family are differentially activated by LPA species. Structure-activity relationship of cloned LPA receptors. *FEBS Lett* **478:**159-165.

Bara I, Ozier A, Tunon de Lara JM, Marthan R and Berger P (2010) Pathophysiology of bronchial smooth muscle remodelling in asthma. *Eur Respir J* **36:**1174-1184.

Barnes PJ and Adcock IM (1997) NF-kappa B: a pivotal role in asthma and a new target for therapy. *Trends Pharmacol Sci* **18:**46-50.

Barnes PJ, Chowdhury B, Kharitonov SA, Magnussen H, Page CP, Postma D and Saetta M (2006) Pulmonary biomarkers in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **174:**6-14.

Barr AJ, Brass LF and Manning DR (1997) Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells. A direct evaluation of selectivity in receptor.G protein coupling. *J Biol Chem* **272:**2223-2229.

Barth MC, Ahluwalia N, Anderson TJ, Hardy GJ, Sinha S, Alvarez-Cardona JA, Pruitt IE, Rhee EP, Colvin RA, Gerszten RE (2009) Kynurenic acid triggers firm arrest of leukocytes to vascular endothelium under flow conditions. *J Biol Chem.* **284**:19189-19195.

Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, Gibson P, Ohta K, O'Byrne P, Pedersen SE, Pizzichini E, Sullivan SD, Wenzel SE and Zar HJ (2008) Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J* **31:**143-178.

Becker PM, Kazi AA, Wadgaonkar R, Pearse DB, Kwiatkowski D and Garcia JG (2003) Pulmonary vascular permeability and ischemic injury in gelsolin-deficient mice. *Am J Respir Cell Mol Biol* **28:**478-484.

Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R and Tunon-de-Lara JM (2007) Inflammation of bronchial smooth muscle in allergic asthma. *Thorax* **62:**8-15.

Benayoun L, Druilhe A, Dombret MC, Aubier M and Pretolani M (2003) Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* **167:**1360-1368.

Benovic JL, DeBlasi A, Stone WC, Caron MG and Lefkowitz RJ (1989) Beta-adrenergic receptor kinase: primary structure delineates a multigene family. *Science* **246:**235-240.

Berair R, Saunders R and Brightling CE (2013) Origins of increased airway smooth muscle mass in asthma. *BMC Med* **11:**145-7015-11-145.

Berridge MJ and Irvine RF (1989) Inositol phosphates and cell signalling. *Nature* **341:**197-205.

Bian D, Mahanivong C, Yu J, Frisch SM, Pan ZK, Ye RD and Huang S (2006) The G12/13-RhoA signaling pathway contributes to efficient lysophosphatidic acid-stimulated cell migration. *Oncogene* **25:**2234-2244.

Bian D, Su S, Mahanivong C, Cheng RK, Han Q, Pan ZK, Sun P and Huang S (2004) Lysophosphatidic Acid Stimulates Ovarian Cancer Cell Migration via a Ras-MEK Kinase 1 Pathway. *Cancer Res* **64:**4209-4217.

Billington CK, Hall IP, Mundell SJ, Parent JL, Panettieri RA,Jr, Benovic JL and Penn RB (1999) Inflammatory and contractile agents sensitize specific adenylyl cyclase isoforms in human airway smooth muscle. *Am J Respir Cell Mol Biol* **21:**597-606.

Billington CK, Ojo OO, Penn RB and Ito S (2012) cAMP regulation of airway smooth muscle function. *Pulm Pharmacol Ther*

Bittman R, Swords B, Liliom K and Tigyi G (1996) Inhibitors of lipid phosphatidate receptors: N-palmitoyl-serine and N-palmitoyl-tyrosine phosphoric acids. *J Lipid Res* **37:**391-398.

Black JL, Roth M, Lee J, Carlin S and Johnson PR (2001) Mechanisms of airway remodeling. Airway smooth muscle. *Am J Respir Crit Care Med* **164:**S63-6.

Bogard AS, Xu C and Ostrom RS (2011) Human bronchial smooth muscle cells express adenylyl cyclase isoforms 2, 4, and 6 in distinct membrane microdomains. *J Pharmacol Exp Ther* **337:**209-217.

Bokoch GM, Katada T, Northup JK, Ui M and Gilman AG (1984) Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. *J Biol Chem* **259:**3560-3567.

Bottema RW, Nolte IM, Howard TD, Koppelman GH, Dubois AE, de Meer G, Kerkhof M, Bleecker ER, Meyers DA and Postma DS (2010) Interleukin 13 and interleukin 4 receptor-alpha polymorphisms in rhinitis and asthma. *Int Arch Allergy Immunol* **153:**259-267.

Bourne HR, Sanders DA and McCormick F (1990) The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* **348:**125-132.

Bousquet J, Jeffery PK, Busse WW, Johnson M and Vignola AM (2000) Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* **161:**1720-1745.

Bousquet J, Mantzouranis E, Cruz AA, Ait-Khaled N, Baena-Cagnani CE, Bleecker ER, Brightling CE, Burney P, Bush A, Busse WW, Casale TB, Chan-Yeung M, Chen R, Chowdhury B, Chung KF, Dahl R, Drazen JM, Fabbri LM, Holgate ST, Kauffmann F, Haahtela T, Khaltaev N, Kiley JP, Masjedi MR, Mohammad Y, O'Byrne P, Partridge MR, Rabe KF, Togias A, van Weel C, Wenzel S, Zhong N and Zuberbier T (2010) Uniform definition of asthma severity, control, and exacerbations: document presented for the World Health Organization Consultation on Severe Asthma. *J Allergy Clin Immunol* **126:**926-938.

Brown BL, Albano JD, Ekins RP and Sgherzi AM (1971) A simple and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. *Biochem J* **121:**561-562.

Brown SL, Jala VR, Raghuwanshi SK, Nasser MW, Haribabu B and Richardson RM (2006) Activation and regulation of platelet-activating factor receptor: role of G(i) and G(q) in receptor-mediated chemotactic, cytotoxic, and cross-regulatory signals. *J Immunol* **177:**3242-3249.

Burrows B, Martinez FD, Halonen M, Barbee RA and Cline MG (1989) Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* **320:**271-277.

Busse WW and Lemanske RF,Jr (2001) Asthma. *N Engl J Med* **344:**350-362.

Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR and Hamm HE (2003) Insights into G protein structure, function, and regulation. *Endocr Rev* **24:**765-781.

Cali JJ, Zwaagstra JC, Mons N, Cooper DM and Krupinski J (1994) Type VIII adenylyl cyclase. A Ca2+/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. *J Biol Chem* **269:**12190-12195.

Caput D, Laurent P, Kaghad M, Lelias JM, Lefort S, Vita N and Ferrara P (1996) Cloning and characterization of a specific interleukin (IL)-13 binding protein structurally related to the IL-5 receptor alpha chain. *J Biol Chem* **271:**16921-16926.

Carlin SM, Roth M and Black JL (2003) Urokinase potentiates PDGF-induced chemotaxis of human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **284:**L1020-6.

Cassel D and Pfeuffer T (1978) Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc Natl Acad Sci U S A* **75:**2669-2673.

Cerione RA, Sibley DR, Codina J, Benovic JL, Winslow J, Neer EJ, Birnbaumer L, Caron MG and Lefkowitz RJ (1984) Reconstitution of a hormone-sensitive adenylate cyclase system. The pure beta-adrenergic receptor and guanine nucleotide regulatory protein confer hormone responsiveness on the resolved catalytic unit. *J Biol Chem* **259:**9979-9982.

Cerutis DR, Nogami M, Anderson JL, Churchill JD, Romberger DJ, Rennard SI and Toews ML (1997) Lysophosphatidic acid and EGF stimulate mitogenesis in human airway smooth muscle cells. *Am J Physiol* **273:**L10-5.

Cerutis DR, Nogami M, Anderson JL, Churchill JD, Romberger DJ, Rennard SI and Toews ML (1997) Lysophosphatidic acid and EGF stimulate mitogenesis in human airway smooth muscle cells. *Am J Physiol* **273:**L10-5.

Chang CL, Lin ME, Hsu HY, Yao CL, Hwang SM, Pan CY, Hsu CY and Lee H (2008) Lysophosphatidic acid-induced interleukin-1 beta expression is mediated through Gi/Rho and the generation of reactive oxygen species in macrophages. *J Biomed Sci* **15:**357-363.

Chen J, Baydoun AR, Xu R, Deng L, Liu X, Zhu W, Shi L, Cong X, Hu S and Chen X (2008) Lysophosphatidic acid protects mesenchymal stem cells against hypoxia and serum deprivation-induced apoptosis. *Stem Cells* **26:**135-145.

Chen J, Chen Y, Zhu W, Han Y, Han B, Xu R, Deng L, Cai Y, Cong X, Yang Y, Hu S and Chen X (2008) Specific LPA receptor subtype mediation of LPA-induced hypertrophy of cardiac myocytes and involvement of Akt and NFkappaB signal pathways. *J Cell Biochem* **103:**1718-1731.

Chen SU, Lee H, Chang DY, Chou CH, Chang CY, Chao KH, Lin CW and Yang YS (2008) Lysophosphatidic acid mediates interleukin-8 expression in human endometrial stromal cells through its receptor and nuclear factor-kappaB-dependent pathway: a possible role in angiogenesis of endometrium and placenta. *Endocrinology* **149:**5888-5896.

Cheng R, Cheng L, Shao MY, Yang H, Wang FM, Hu T and Zhou XD (2010) Roles of lysophosphatidic acid and the Rho-associated kinase pathway in the migration of dental pulp cells. *Exp Cell Res* **316:**1019-1027.

Chettibi S, Lawrence AJ, Stevenson RD and Young JD (1994) Effect of lysophosphatidic acid on motility, polarisation and metabolic burst of human neutrophils. *FEMS Immunol Med Microbiol* **8:**271-281.

Cheung D, Timmers MC, Zwinderman AH, Bel EH, Dijkman JH and Sterk PJ (1992) Long-term effects of a long-acting beta 2-adrenoceptor agonist, salmeterol, on airway hyperresponsiveness in patients with mild asthma. *N Engl J Med* **327:**1198-1203.

Chiono M, Mahey R, Tate G and Cooper DM (1995) Capacitative Ca2+ entry exclusively inhibits cAMP synthesis in C6-2B glioma cells. Evidence that physiologically evoked Ca2+ entry regulates Ca(2+)-inhibitable adenylyl cyclase in non-excitable cells. *J Biol Chem* **270:**1149-1155.

Chiu TT, Leung WY, Moyer MP, Strieter RM and Rozengurt E (2007) Protein kinase D2 mediates lysophosphatidic acid-induced interleukin 8 production in nontransformed human colonic epithelial cells through NF-kappaB. *Am J Physiol Cell Physiol* **292:**C767-77.

Choi EJ, Xia Z and Storm DR (1992) Stimulation of the type III olfactory adenylyl cyclase by calcium and calmodulin. *Biochemistry* **31:**6492-6498.

Choi JW, Herr DR, Noguchi K, Yung YC, Lee CW, Mutoh T, Lin ME, Teo ST, Park KE, Mosley AN and Chun J (2010) LPA receptors: subtypes and biological actions. *Annu Rev Pharmacol Toxicol* **50:**157-186.

Choi JW, Lee CW and Chun J (2008) Biological roles of lysophospholipid receptors revealed by genetic null mice: an update. *Biochim Biophys Acta* **1781:**531-539.

Christofidou-Solomidou M, Scherpereel A, Solomides CC, Muzykantov VR, Machtay M, Albelda SM and DiNubile MJ (2002) Changes in plasma gelsolin concentration during acute oxidant lung injury in mice. *Lung* **180:**91-104.

Chun J, Goetzl EJ, Hla T, Igarashi Y, Lynch KR, Moolenaar W, Pyne S and Tigyi G (2002) International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol Rev* **54:**265-269.

Chun J, Hla T, Lynch KR, Spiegel S and Moolenaar WH (2010) International Union of Basic and Clinical Pharmacology. LXXVIII. Lysophospholipid receptor nomenclature. *Pharmacol Rev* **62:**579-587.

Chung KF and Barnes PJ (1999) Cytokines in asthma. *Thorax* **54:**825-857.

Contos JJ and Chun J (2000) Genomic characterization of the lysophosphatidic acid receptor gene, lp(A2)/Edg4, and identification of a frameshift mutation in a previously characterized cDNA. *Genomics* **64:**155-169.

Contos JJ, Fukushima N, Weiner JA, Kaushal D and Chun J (2000) Requirement for the lpA1 lysophosphatidic acid receptor gene in normal suckling behavior. *Proc Natl Acad Sci U S A* **97:**13384-13389.

Contos JJ, Ishii I and Chun J (2000) Lysophosphatidic acid receptors. *Mol Pharmacol* **58:**1188-1196.

Contos JJ, Ishii I, Fukushima N, Kingsbury MA, Ye X, Kawamura S, Brown JH and Chun J (2002) Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2). *Mol Cell Biol* **22:**6921-6929.

Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, Harris JM, Scheerens H, Wu LC, Su Z, Mosesova S, Eisner MD, Bohen SP and Matthews JG (2011) Lebrikizumab treatment in adults with asthma. *N Engl J Med* **365:**1088-1098.

Craft CM, Whitmore DH and Wiechmann AF (1994) Cone arrestin identified by targeting expression of a functional family. *J Biol Chem* **269:**4613-4619.

Cremers B, Flesch M, Kostenis E, Maack C, Niedernberg A, Stoff A, Sudkamp M, Wendler O and Bohm M (2003) Modulation of myocardial contractility by lysophosphatidic acid (LPA). *J Mol Cell Cardiol* **35:**71-80.

Cui MZ, Zhao G, Winokur AL, Laag E, Bydash JR, Penn MS, Chisolm GM and Xu X (2003) Lysophosphatidic acid induction of tissue factor expression in aortic smooth muscle cells. *Arterioscler Thromb Vasc Biol* **23:**224-230.

Cummings R, Zhao Y, Jacoby D, Spannhake EW, Ohba M, Garcia JG, Watkins T, He D, Saatian B and Natarajan V (2004) Protein kinase Cdelta mediates lysophosphatidic acid-induced NF-kappaB activation and interleukin-8 secretion in human bronchial epithelial cells. *J Biol Chem* **279:**41085-41094.

Dahl B, Schiodt FV, Ott P, Gvozdenovic R, Yin HL and Lee WM (1999) Plasma gelsolin is reduced in trauma patients. *Shock* **12:**102-104.

Davenport AP, Alexander SP, Sharman JL, Pawson AJ, Benson HE, Monaghan AE, Liew WC, Mpamhanga CP, Bonner TI, Neubig RR, Pin JP, Spedding M and Harmar AJ (2013) International Union of Basic and Clinical Pharmacology. LXXXVIII. G protein-coupled receptor list: recommendations for new pairings with cognate ligands. *Pharmacol Rev* **65:**967-986.

Demoyer JS, Skalak TC and Durieux ME (2000) Lysophosphatidic acid enhances healing of acute cutaneous wounds in the mouse. *Wound Repair Regen* **8:**530-537.

Deng W, Balazs L, Wang DA, Van Middlesworth L, Tigyi G and Johnson LR (2002) Lysophosphatidic acid protects and rescues intestinal epithelial cells from radiation- and chemotherapy-induced apoptosis. *Gastroenterology* **123:**206-216.

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G and Zumla A (2004) Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *BioTechniques* **37:**112-4, 116, 118-9.

Dutt P, Nguyen N and Toksoz D (2004) Role of Lbc RhoGEF in Galpha12/13-induced signals to Rho GTPase. *Cell Signal* **16:**201-209.

Dutt P, Wang JF and Groopman JE (1998) Stromal cell-derived factor-1 alpha and stem cell factor/kit ligand share signaling pathways in hemopoietic progenitors: a potential mechanism for cooperative induction of chemotaxis. *J Immunol* **161:**3652-3658.

Ediger TL, Danforth BL and Toews ML (2002) Lysophosphatidic acid upregulates the epidermal growth factor receptor in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **282:**L91-8.

Ediger TL, Schulte NA, Murphy TJ and Toews ML (2003) Transcription factor activation and mitogenic synergism in airway smooth muscle cells. *Eur Respir J* **21:**759-769.

Ediger TL and Toews ML (2001) Dual effects of lysophosphatidic acid on human airway smooth muscle cell proliferation and survival. *Biochim Biophys Acta* **1531:**59-67.

Ediger TL and Toews ML (2001) Dual effects of lysophosphatidic acid on human airway smooth muscle cell proliferation and survival. *Biochim Biophys Acta* **1531:**59-67.

Ediger TL and Toews ML (2000) Synergistic stimulation of airway smooth muscle cell mitogenesis. *J Pharmacol Exp Ther* **294:**1076-1082.

Eichholtz T, Jalink K, Fahrenfort I and Moolenaar WH (1993) The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem J* **291 (Pt 3):**677-680.

Emala C, Black C, Curry C, Levine MA and Hirshman CA (1993) Impaired beta-adrenergic receptor activation of adenylyl cyclase in airway smooth muscle in the basenji-greyhound dog model of airway hyperresponsiveness. *Am J Respir Cell Mol Biol* **8:**668-675.

Emo J, Meednu N, Chapman TJ, Rezaee F, Balys M, Randall T, Rangasamy T and Georas SN (2012) Lpa2 is a negative regulator of both dendritic cell activation and murine models of allergic lung inflammation. *J Immunol* **188:**3784-3790.

Erickson JR, Wu JJ, Goddard JG, Tigyi G, Kawanishi K, Tomei LD and Kiefer MC (1998) Edg-2/Vzg-1 couples to the yeast pheromone response pathway selectively in response to lysophosphatidic acid. *J Biol Chem* **273:**1506-1510.

Estivill-Torrus G, Llebrez-Zayas P, Matas-Rico E, Santin L, Pedraza C, De Diego I, Del Arco I, Fernandez-Llebrez P, Chun J and De Fonseca FR (2008) Absence of LPA1 signaling results in defective cortical development. *Cereb Cortex* **18:**938-950.

Fan H, Zingarelli B, Harris V, Tempel GE, Halushka PV and Cook JA (2008) Lysophosphatidic acid inhibits bacterial endotoxin-induced pro-inflammatory response: potential anti-inflammatory signaling pathways. *Mol Med* **14:**422-428.

Fang X, Schummer M, Mao M, Yu S, Tabassam FH, Swaby R, Hasegawa Y, Tanyi JL, LaPushin R, Eder A, Jaffe R, Erickson J and Mills GB (2002) Lysophosphatidic acid is a bioactive mediator in ovarian cancer. *Biochim Biophys Acta* **1582:**257-264.

Federman AD, Conklin BR, Schrader KA, Reed RR and Bourne HR (1992) Hormonal stimulation of adenylyl cyclase through Gi-protein beta gamma subunits. *Nature* **356:**159-161.

Fitzpatrick AM, Stephenson ST, Hadley GR, Burwell L, Penugonda M, Simon DM, Hansen J, Jones DP and Brown LA (2011) Thiol redox disturbances in children with severe asthma are associated with posttranslational modification of the transcription factor nuclear factor (erythroid-derived 2)-like 2. *J Allergy Clin Immunol* **127:**1604-1611.

Flower DR (1999) Modelling G-protein-coupled receptors for drug design. *Biochim Biophys Acta* **1422:**207-234.

Fox MA, Colello RJ, Macklin WB and Fuss B (2003) Phosphodiesterase-Ialpha/autotaxin: a counteradhesive protein expressed by oligodendrocytes during onset of myelination. *Mol Cell Neurosci* **23:**507-519.

Fredriksson R, Lagerstrom MC, Lundin LG and Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* **63:**1256-1272.

Fueller M, Wang DA, Tigyi G and Siess W (2003) Activation of human monocytic cells by lysophosphatidic acid and sphingosine-1-phosphate. *Cell Signal* **15:**367-375.

Fukuhara S, Chikumi H and Gutkind JS (2000) Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho. *FEBS Lett* **485:**183-188.

Fukuhara S, Murga C, Zohar M, Igishi T and Gutkind JS (1999) A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. *J Biol Chem* **274:**5868-5879.

Fukushima N, Kimura Y and Chun J (1998) A single receptor encoded by vzg-1/lpA1/edg-2 couples to G proteins and mediates multiple cellular responses to lysophosphatidic acid. *Proc Natl Acad Sci U S A* **95:**6151-6156.

Fukushima N, Shano S, Moriyama R and Chun J (2007) Lysophosphatidic acid stimulates neuronal differentiation of cortical neuroblasts through the LPA1-G(i/o) pathway. *Neurochem Int* **50:**302-307.

Gage RM, Matveeva EA, Whiteheart SW and von Zastrow M (2005) Type I PDZ ligands are sufficient to promote rapid recycling of G Protein-coupled receptors independent of binding to N-ethylmaleimide-sensitive factor. *J Biol Chem* **280:**3305-3313.

Ganguly K, Stoeger T, Wesselkamper SC, Reinhard C, Sartor MA, Medvedovic M, Tomlinson CR, Bolle I, Mason JM, Leikauf GD and Schulz H (2007) Candidate genes controlling pulmonary function in mice: transcript profiling and predicted protein structure. *Physiol Genomics* **31:**410-421.

Garcia-Mata R, Boulter E and Burridge K (2011) The 'invisible hand': regulation of RHO GTPases by RHOGDIs. *Nat Rev Mol Cell Biol* **12:**493-504.

Gauvreau GM, Watson RM and O'Byrne PM (1999) Protective effects of inhaled PGE2 on allergen-induced airway responses and airway inflammation. *Am J Respir Crit Care Med* **159:**31-36.

Georas SN, Berdyshev E, Hubbard W, Gorshkova IA, Usatyuk PV, Saatian B, Myers AC, Williams MA, Xiao HQ, Liu M and Natarajan V (2007) Lysophosphatidic acid is detectable in human bronchoalveolar lavage fluids at baseline and increased after segmental allergen challenge. *Clin Exp Allergy* **37:**311-322.

Gerrard JM, Kindom SE, Peterson DA, Peller J, Krantz KE and White JG (1979) Lysophosphatidic acids. Influence on platelet aggregation and intracellular calcium flux. *Am J Pathol* **96:**423-438.

Gesty-Palmer D, Flannery P, Yuan L, Corsino L, Spurney R, Lefkowitz RJ and Luttrell LM (2009) A beta-arrestin-biased agonist of the parathyroid hormone receptor (PTH1R) promotes bone formation independent of G protein activation. *Sci Transl Med* **1:**1ra1.

Giembycz MA and Newton R (2011) Harnessing the clinical efficacy of phosphodiesterase 4 inhibitors in inflammatory lung diseases: dual-selective phosphodiesterase inhibitors and novel combination therapies. *Handb Exp Pharmacol* **(204):**415-446.

Gierse J, Thorarensen A, Beltey K, Bradshaw-Pierce E, Cortes-Burgos L, Hall T, Johnston A, Murphy M, Nemirovskiy O, Ogawa S, Pegg L, Pelc M, Prinsen M, Schnute M, Wendling J, Wene S, Weinberg R, Wittwer A, Zweifel B and Masferrer J (2010) A novel autotaxin inhibitor reduces lysophosphatidic acid levels in plasma and the site of inflammation. *J Pharmacol Exp Ther* **334:**310-317.

Giganti A, Rodriguez M, Fould B, Moulharat N, Coge F, Chomarat P, Galizzi JP, Valet P, Saulnier-Blache JS, Boutin JA and Ferry G (2008) Murine and human autotaxin alpha, beta, and gamma isoforms: gene organization, tissue distribution, and biochemical characterization. *J Biol Chem* **283:**7776-7789.

Gilchrist A, Vanhauwe JF, Li A, Thomas TO, Voyno-Yasenetskaya T and Hamm HE (2001) G alpha minigenes expressing C-terminal peptides serve as specific inhibitors of thrombin-mediated endothelial activation. *J Biol Chem* **276:**25672-25679.

Gill DM and Meren R (1978) ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. *Proc Natl Acad Sci U S A* **75:**3050-3054.

GINA report (2012) From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2012. Available from: http://www.ginasthma.org/. **2013:**110.

Gizycki MJ, Adelroth E, Rogers AV, O'Byrne PM and Jeffery PK (1997) Myofibroblast involvement in the allergen-induced late response in mild atopic asthma. *Am J Respir Cell Mol Biol* **16:**664-673.

Goetzl EJ, Dolezalova H, Kong Y, Hu YL, Jaffe RB, Kalli KR and Conover CA (1999) Distinctive expression and functions of the type 4 endothelial differentiation gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer. *Cancer Res* **59:**5370-5375.

Goetzl EJ, Kong Y and Mei B (1999) Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. *J Immunol* **162:**2049-2056.

Goetzl EJ, Lee H, Azuma T, Stossel TP, Turck CW and Karliner JS (2000) Gelsolin binding and cellular presentation of lysophosphatidic acid. *J Biol Chem* **275:**14573-14578.

Golla R and Seethala R (2002) A homogeneous enzyme fragment complementation cyclic AMP screen for GPCR agonists. *J Biomol Screen* **7:**515-525.

Goncharova EA, Billington CK, Irani C, Vorotnikov AV, Tkachuk VA, Penn RB, Krymskaya VP and Panettieri RA,Jr (2003) Cyclic AMP-mobilizing agents and glucocorticoids modulate human smooth muscle cell migration. *Am J Respir Cell Mol Biol* **29:**19-27.

Goncharova EA, Billington CK, Irani C, Vorotnikov AV, Tkachuk VA, Penn RB, Krymskaya VP and Panettieri RA,Jr (2003) Cyclic AMP-mobilizing agents and glucocorticoids modulate human smooth muscle cell migration. *Am J Respir Cell Mol Biol* **29:**19-27.

Govindaraju V, Michoud MC, Al-Chalabi M, Ferraro P, Powell WS and Martin JG (2006) Interleukin-8: novel roles in human airway smooth muscle cell contraction and migration. *Am J Physiol Cell Physiol* **291:**C957-65.

Graler MH and Goetzl EJ (2002) Lysophospholipids and their G protein-coupled receptors in inflammation and immunity. *Biochim Biophys Acta* **1582:**168-174.

Guillou JL, Nakata H and Cooper DM (1999) Inhibition by calcium of mammalian adenylyl cyclases. *J Biol Chem* **274:**35539-35545.

Gustin C, Delaive E, Dieu M, Calay D and Raes M (2008) Upregulation of pentraxin-3 in human endothelial cells after lysophosphatidic acid exposure. *Arterioscler Thromb Vasc Biol* **28:**491-497.

Gustin C, Van Steenbrugge M and Raes M (2008) LPA modulates monocyte migration directly and via LPA-stimulated endothelial cells. *Am J Physiol Cell Physiol* **295:**C905-14.

Hains MD, Wing MR, Maddileti S, Siderovski DP and Harden TK (2006) Galpha12/13- and rho-dependent activation of phospholipase C-epsilon by lysophosphatidic acid and thrombin receptors. *Mol Pharmacol* **69:**2068-2075.

Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, Wardlaw AJ and Green RH (2008) Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med* **178:**218-224.

Halder SK, Yano R, Chun J and Ueda H (2013) Involvement of LPA1 receptor signaling in cerebral ischemia-induced neuropathic pain. *Neuroscience* **235:**10-15.

Haley KJ, Sunday ME, Wiggs BR, Kozakewich HP, Reilly JJ, Mentzer SJ, Sugarbaker DJ, Doerschuk CM and Drazen JM (1998) Inflammatory cell distribution within and along asthmatic airways. *Am J Respir Crit Care Med* **158:**565-572.

Hall RA, Ostedgaard LS, Premont RT, Blitzer JT, Rahman N, Welsh MJ and Lefkowitz RJ (1998) A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na+/H+ exchanger regulatory factor family of PDZ proteins. *Proc Natl Acad Sci U S A* **95:**8496-8501.

Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A, Stoffel RH, Barak LS, Shenolikar S, Weinman EJ, Grinstein S and Lefkowitz RJ (1998) The beta2-adrenergic receptor interacts with the Na+/H+-exchanger regulatory factor to control Na+/H+ exchange. *Nature* **392:**626-630.

Hama K, Aoki J, Fukaya M, Kishi Y, Sakai T, Suzuki R, Ohta H, Yamori T, Watanabe M, Chun J and Arai H (2004) Lysophosphatidic acid and autotaxin stimulate cell motility of neoplastic and non-neoplastic cells through LPA1. *J Biol Chem* **279:**17634-17639.

Hama K, Aoki J, Inoue A, Endo T, Amano T, Motoki R, Kanai M, Ye X, Chun J, Matsuki N, Suzuki H, Shibasaki M and Arai H (2007) Embryo spacing and implantation timing are differentially regulated by LPA3-mediated lysophosphatidic acid signaling in mice. *Biol Reprod* **77:**954-959.

Hama K, Bandoh K, Kakehi Y, Aoki J and Arai H (2002) Lysophosphatidic acid (LPA) receptors are activated differentially by biological fluids: possible role of LPA-binding proteins in activation of LPA receptors. *FEBS Lett* **523:**187-192.

Hansen G, Jin S, Umetsu DT and Conti M (2000) Absence of muscarinic cholinergic airway responses in mice deficient in the cyclic nucleotide phosphodiesterase PDE4D. *Proc Natl Acad Sci U S A* **97:**6751-6756.

Harrison SM, Reavill C, Brown G, Brown JT, Cluderay JE, Crook B, Davies CH, Dawson LA, Grau E, Heidbreder C, Hemmati P, Hervieu G, Howarth A, Hughes ZA, Hunter AJ, Latcham J, Pickering S, Pugh P, Rogers DC, Shilliam CS and Maycox PR (2003) LPA1 receptor-deficient mice have phenotypic changes observed in psychiatric disease. *Mol Cell Neurosci* **24:**1170-1179.

Hasegawa Y, Erickson JR, Goddard GJ, Yu S, Liu S, Cheng KW, Eder A, Bandoh K, Aoki J, Jarosz R, Schrier AD, Lynch KR, Mills GB and Fang X (2003) Identification of a phosphothionate analogue of lysophosphatidic acid (LPA) as a selective agonist of the LPA3 receptor. *J Biol Chem* **278:**11962-11969.

Hashimoto T, Nakano Y, Ohata H and Momose K (2001) Lysophosphatidic acid enhances airway response to acetylcholine in guinea pigs. *Life Sci* **70:**199-205.

Hashimoto T, Nakano Y, Yamashita M, Fang YI, Ohata H and Momose K (2002) Role of Rho-associated protein kinase and histamine in lysophosphatidic acid-induced airway hyperresponsiveness in guinea pigs. *Jpn J Pharmacol* **88:**256-261.

Hashimoto T, Yamashita M, Ohata H and Momose K (2003) Lysophosphatidic acid enhances in vivo infiltration and activation of guinea pig eosinophils and neutrophils via a Rho/Rho-associated protein kinase-mediated pathway. *J Pharmacol Sci* **91:**8-14.

Hausmann J, Kamtekar S, Christodoulou E, Day JE, Wu T, Fulkerson Z, Albers HM, van Meeteren LA, Houben AJ, van Zeijl L, Jansen S, Andries M, Hall T, Pegg LE, Benson TE, Kasiem M, Harlos K, Kooi CW, Smyth SS, Ovaa H, Bollen M, Morris AJ, Moolenaar WH and Perrakis A (2011) Structural basis of substrate discrimination and integrin binding by autotaxin. *Nat Struct Mol Biol* **18:**198-204.

Hausmann J, Perrakis A and Moolenaar WH (2013) Structure-function relationships of autotaxin, a secreted lysophospholipase D. *Adv Biol Regul* **53:**112-117.

Hayashi K, Takahashi M, Nishida W, Yoshida K, Ohkawa Y, Kitabatake A, Aoki J, Arai H and Sobue K (2001) Phenotypic modulation of vascular smooth muscle cells induced by unsaturated lysophosphatidic acids. *Circ Res* **89:**251-258.

Hayashi M, Okabe K, Yamawaki Y, Teranishi M, Honoki K, Mori T, Fukushima N and Tsujiuchi T (2011) Loss of lysophosphatidic acid receptor-3 enhances cell migration in rat lung tumor cells. *Biochem Biophys Res Commun*

He D, Natarajan V, Stern R, Gorshkova IA, Solway J, Spannhake EW and Zhao Y (2008) Lysophosphatidic acid-induced transactivation of epidermal growth factor receptor regulates cyclo-oxygenase-2 expression and prostaglandin E(2) release via C/EBPbeta in human bronchial epithelial cells. *Biochem J* **412:**153-162.

He D, Su Y, Usatyuk PV, Spannhake EW, Kogut P, Solway J, Natarajan V and Zhao Y (2009) Lysophosphatidic acid enhances pulmonary epithelial barrier integrity and protects endotoxin-induced epithelial barrier disruption and lung injury. *J Biol Chem* **284:**24123-24132.

Heasley BH, Jarosz R, Lynch KR and Macdonald TL (2004) Initial structure-activity relationships of lysophosphatidic acid receptor antagonists: discovery of a high-affinity LPA1/LPA3 receptor antagonist. *Bioorg Med Chem Lett* **14:**2735-2740.

Hecht JH, Weiner JA, Post SR and Chun J (1996) Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J Cell Biol* **135:**1071-1083.

Hedges JC, Dechert MA, Yamboliev IA, Martin JL, Hickey E, Weber LA and Gerthoffer WT (1999) A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. *J Biol Chem* **274:**24211-24219.

Heise CE, Santos WL, Schreihofer AM, Heasley BH, Mukhin YV, Macdonald TL and Lynch KR (2001) Activity of 2-substituted lysophosphatidic acid (LPA) analogs at LPA receptors: discovery of a LPA1/LPA3 receptor antagonist. *Mol Pharmacol* **60:**1173-1180.

Hill SJ, Williams C and May LT (2010) Insights into GPCR pharmacology from the measurement of changes in intracellular cyclic AMP; advantages and pitfalls of differing methodologies. *Br J Pharmacol* **161:**1266-1275.

Hirakawa M, Karashima Y, Watanabe M, Kimura C, Ito Y and Oike M (2007) Protein kinase A inhibits lysophosphatidic acid-induced migration of airway smooth muscle cells. *J Pharmacol Exp Ther* **321:**1102-1108.

Hirakawa M, Karashima Y, Watanabe M, Kimura C, Ito Y and Oike M (2007) Protein kinase A inhibits lysophosphatidic acid-induced migration of airway smooth muscle cells. *J Pharmacol Exp Ther* **321:**1102-1108.

Hiramatsu T, Sonoda H, Takanezawa Y, Morikawa R, Ishida M, Kasahara K, Sanai Y, Taguchi R, Aoki J and Arai H (2003) Biochemical and molecular characterization of two phosphatidic acid-selective phospholipase A1s, mPA-PLA1alpha and mPA-PLA1beta. *J Biol Chem* **278:**49438-49447.

Hirshman CA and Emala CW (1999) Actin reorganization in airway smooth muscle cells involves Gq and Gi-2 activation of Rho. *Am J Physiol* **277:**L653-61.

Holz GG,4th, Rane SG and Dunlap K (1986) GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature* **319:**670-672.

Hooks SB, Ragan SP, Hopper DW, Honemann CW, Durieux ME, Macdonald TL and Lynch KR (1998) Characterization of a receptor subtype-selective lysophosphatidic acid mimetic. *Mol Pharmacol* **53:**188-194.

Hooks SB, Ragan SP and Lynch KR (1998) Identification of a novel human phosphatidic acid phosphatase type 2 isoform. *FEBS Lett* **427:**188-192.

Hope JM, Wang FQ, Whyte JS, Ariztia EV, Abdalla W, Long K and Fishman DA (2009) LPA receptor 2 mediates LPA-induced endometrial cancer invasion. *Gynecol Oncol* **112:**215-223.

Houslay MD and Adams DR (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem J* **370:**1-18.

Huggett J, Dheda K, Bustin S and Zumla A (2005) Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun* **6:**279-284.

Idzko M, Laut M, Panther E, Sorichter S, Durk T, Fluhr JW, Herouy Y, Mockenhaupt M, Myrtek D, Elsner P and Norgauer J (2004) Lysophosphatidic acid induces chemotaxis, oxygen radical production, CD11b up-regulation, Ca2+ mobilization, and actin reorganization in human eosinophils via pertussis toxin-sensitive G proteins. *J Immunol* **172:**4480-4485.

Im DS, Heise CE, Harding MA, George SR, O'Dowd BF, Theodorescu D and Lynch KR (2000) Molecular cloning and characterization of a lysophosphatidic acid receptor, Edg-7, expressed in prostate. *Mol Pharmacol* **57:**753-759.

Ingram JL and Kraft M (2012) IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *J Allergy Clin Immunol* **130:**829-42; quiz 843-4.

Inoue A, Arima N, Ishiguro J, Prestwich GD, Arai H and Aoki J (2011) LPA-producing enzyme PA-PLA(1)alpha regulates hair follicle development by modulating EGFR signalling. *EMBO J* **30:**4248-4260.

Ishii I, Contos JJ, Fukushima N and Chun J (2000) Functional comparisons of the lysophosphatidic acid receptors, LP(A1)/VZG-1/EDG-2, LP(A2)/EDG-4, and

LP(A3)/EDG-7 in neuronal cell lines using a retrovirus expression system. *Mol Pharmacol* **58:**895-902.

Ishii I, Fukushima N, Ye X and Chun J (2004) Lysophospholipid receptors: signaling and biology. *Annu Rev Biochem* **73:**321-354.

Itagaki K, Kannan KB and Hauser CJ (2005) Lysophosphatidic acid triggers calcium entry through a non-store-operated pathway in human neutrophils. *J Leukoc Biol* **77:**181-189.

Iwanicki MP, Vomastek T, Tilghman RW, Martin KH, Banerjee J, Wedegaertner PB and Parsons JT (2008) FAK, PDZ-RhoGEF and ROCKII cooperate to regulate adhesion movement and trailing-edge retraction in fibroblasts. *J Cell Sci* **121:**895-905.

Iyoda T, Zhang F, Sun L, Hao F, Schmitz-Peiffer C, Xu X and Cui MZ (2012) Lysophosphatidic acid induces early growth response-1 (Egr-1) protein expression via protein kinase Cdelta-regulated extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) activation in vascular smooth muscle cells. *J Biol Chem* **287:**22635-22642.

Jalink K, Eichholtz T, Postma FR, van Corven EJ and Moolenaar WH (1993) Lysophosphatidic acid induces neuronal shape changes via a novel, receptor-mediated signaling pathway: similarity to thrombin action. *Cell Growth Differ* **4:**247-255.

Jalink K, Hengeveld T, Mulder S, Postma FR, Simon MF, Chap H, van der Marel GA, van Boom JH, van Blitterswijk WJ and Moolenaar WH (1995) Lysophosphatidic acid-induced Ca2+ mobilization in human A431 cells: structure-activity analysis. *Biochem J* **307 (Pt 2):**609-616.

James AL, Elliot JG, Jones RL, Carroll ML, Mauad T, Bai TR, Abramson MJ, McKay KO and Green FH (2012) Airway smooth muscle hypertrophy and hyperplasia in asthma. *Am J Respir Crit Care Med* **185:**1058-1064.

Jan CR, Lu YC, Jiann BP, Chang HT, Wang JL, Chen WC and Huang JK (2003) Novel effect of N-palmitoyl-L-serine phosphoric acid on cytosolic Ca2+ levels in human osteoblasts. *Pharmacol Toxicol* **93:**71-76.

Jasinska R, Zhang QX, Pilquil C, Singh I, Xu J, Dewald J, Dillon DA, Berthiaume LG, Carman GM, Waggoner DW and Brindley DN (1999) Lipid phosphate phosphohydrolase-1 degrades exogenous glycerolipid and sphingolipid phosphate esters. *Biochem J* **340 (Pt 3):**677-686.

Jin SL and Conti M (2002) Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF-alpha responses. *Proc Natl Acad Sci U S A* **99:**7628-7633.

Jin SL, Lan L, Zoudilova M and Conti M (2005) Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages. *J Immunol* **175:**1523-1531.

Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, Burgess JK and Black JL (2001) Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* **164:**474-477.

Jongsma M, Matas-Rico E, Rzadkowski A, Jalink K and Moolenaar WH (2011) LPA is a chemorepellent for B16 melanoma cells: action through the cAMP-elevating LPA5 receptor. *PLoS One* **6:**e29260.

Joubert P, Lajoie-Kadoch S, Labonte I, Gounni AS, Maghni K, Wellemans V, Chakir J, Laviolette M, Hamid Q and Lamkhioued B (2005) CCR3 expression and function in asthmatic airway smooth muscle cells. *J Immunol* **175:**2702-2708.

Kach J, Sethakorn N and Dulin NO (2012) A finer tuning of G-protein signaling through regulated control of RGS proteins. *Am J Physiol Heart Circ Physiol* **303:**H19-35.

Kanda H, Newton R, Klein R, Morita Y, Gunn MD and Rosen SD (2008) Autotaxin, an ectoenzyme that produces lysophosphatidic acid, promotes the entry of lymphocytes into secondary lymphoid organs. *Nat Immunol* **9:**415-423.

Kato K, Yoshikawa K, Tanabe E, Kitayoshi M, Fukui R, Fukushima N and Tsujiuchi T (2012) Opposite roles of LPA1 and LPA3 on cell motile and invasive activities of pancreatic cancer cells. *Tumour Biol* **33:**1739-1744.

Katsushima Y, Sato T, Yamada C, Ito M, Suzuki Y, Ogawa E, Sukegawa I, Sukegawa J, Fukunaga K and Yanagisawa T (2013) Interaction of PICK1 with C-Terminus of Growth Hormone-Releasing Hormone Receptor (GHRHR) Modulates Trafficking and Signal Transduction of Human GHRHR. *J Pharmacol Sci* **122:**193-204.

Kaur D, Saunders R, Berger P, Siddiqui S, Woodman L, Wardlaw A, Bradding P and Brightling CE (2006) Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma. *Am J Respir Crit Care Med* **174:**1179-1188.

Kazantseva A, Goltsov A, Zinchenko R, Grigorenko AP, Abrukova AV, Moliaka YK, Kirillov AG, Guo Z, Lyle S, Ginter EK and Rogaev EI (2006) Human hair growth deficiency is linked to a genetic defect in the phospholipase gene LIPH. *Science* **314:**982-985.

Keravis T and Lugnier C (2012) Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: benefits of PDE inhibitors in various diseases and perspectives for future therapeutic developments. *Br J Pharmacol* **165:**1288-1305.

Khandoga AL, Fujiwara Y, Goyal P, Pandey D, Tsukahara R, Bolen A, Guo H, Wilke N, Liu J, Valentine WJ, Durgam GG, Miller DD, Jiang G, Prestwich GD, Tigyi G and Siess W (2008) Lysophosphatidic acid-induced platelet shape change revealed through LPA(1-5) receptor-selective probes and albumin. *Platelets* **19:**415-427.

Khandoga AL, Pandey D, Welsch U, Brandl R and Siess W (2011) GPR92/LPA(5) lysophosphatidate receptor mediates megakaryocytic cell shape change induced by human atherosclerotic plaques. *Cardiovasc Res* **90:**157-164.

Kim EK, Park JM, Lim S, Choi JW, Kim HS, Seok H, Seo JK, Oh K, Lee DS, Kim KT, Ryu SH and Suh PG (2011) Activation of AMP-activated protein kinase is essential for

lysophosphatidic acid-induced cell migration in ovarian cancer cells. *J Biol Chem* **286:**24036-24045.

Kim EK, Yun SJ, Do KH, Kim MS, Cho M, Suh DS, Kim CD, Kim JH, Birnbaum MJ and Bae SS (2008) Lysophosphatidic acid induces cell migration through the selective activation of Akt1. *Exp Mol Med* **40:**445-452.

Kim J, Keys JR and Eckhart AD (2006) Vascular smooth muscle migration and proliferation in response to lysophosphatidic acid (LPA) is mediated by LPA receptors coupling to Gq. *Cell Signal* **18:**1695-1701.

Kirkham S, Sheehan JK, Knight D, Richardson PS and Thornton DJ (2002) Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. *Biochem J* **361:**537-546.

Knight DA and Holgate ST (2003) The airway epithelium: structural and functional properties in health and disease. *Respirology* **8:**432-446.

Koch WJ, Hawes BE, Allen LF and Lefkowitz RJ (1994) Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G beta gamma activation of p21ras. *Proc Natl Acad Sci U S A* **91:**12706-12710.

Kolakowski LF,Jr (1994) GCRDb: a G-protein-coupled receptor database. *Receptors Channels* **2:**1-7.

Komachi M, Damirin A, Malchinkhuu E, Mogi C, Tobo M, Ohta H, Sato K, Tomura H and Okajima F (2009) Signaling pathways involved in DNA synthesis and migration in response to lysophosphatidic acid and low-density lipoprotein in coronary artery smooth muscle cells. *Vascul Pharmacol* **50:**178-184.

Komachi M, Damirin A, Malchinkhuu E, Mogi C, Tobo M, Ohta H, Sato K, Tomura H and Okajima F (2009) Signaling pathways involved in DNA synthesis and migration in response to lysophosphatidic acid and low-density lipoprotein in coronary artery smooth muscle cells. *Vascul Pharmacol* **50:**178-184.

Komachi M, Tomura H, Malchinkhuu E, Tobo M, Mogi C, Yamada T, Kimura T, Kuwabara A, Ohta H, Im DS, Kurose H, Takeyoshi I, Sato K and Okajima F (2009) LPA1 receptors mediate stimulation, whereas LPA2 receptors mediate inhibition, of migration of pancreatic cancer cells in response to lysophosphatidic acid and malignant ascites. *Carcinogenesis* **30:**457-465.

Kotarsky K, Boketoft A, Bristulf J, Nilsson NE, Norberg A, Hansson S, Owman C, Sillard R, Leeb-Lundberg LM and Olde B (2006) Lysophosphatidic acid binds to and activates GPR92, a G protein-coupled receptor highly expressed in gastrointestinal lymphocytes. *J Pharmacol Exp Ther* **318:**619-628.

Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G and Sternweis PC (1998) p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. *Science* **280:**2109-2111.

Kraft M, Djukanovic R, Wilson S, Holgate ST and Martin RJ (1996) Alveolar tissue inflammation in asthma. *Am J Respir Crit Care Med* **154:**1505-1510.

Kremer AE, Martens JJ, Kulik W, Rueff F, Kuiper EM, van Buuren HR, van Erpecum KJ, Kondrackiene J, Prieto J, Rust C, Geenes VL, Williamson C, Moolenaar WH, Beuers U and Oude Elferink RP (2010) Lysophosphatidic acid is a potential mediator of cholestatic pruritus. *Gastroenterology* **139:**1008-18, 1018.e1.

Kreps DM, Whittle SM, Hoffman JM and Toews ML (1993) Lysophosphatidic acid mimics serum-induced sensitization of cyclic AMP accumulation. *FASEB J* **7:**1376-1380.

Krupnick JG, Gurevich VV and Benovic JL (1997) Mechanism of quenching of phototransduction. Binding competition between arrestin and transducin for phosphorhodopsin. *J Biol Chem* **272:**18125-18131.

Kurosu H, Maehama T, Okada T, Yamamoto T, Hoshino S, Fukui Y, Ui M, Hazeki O and Katada T (1997) Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110beta is synergistically activated by the betagamma subunits of G proteins and phosphotyrosyl peptide. *J Biol Chem* **272:**24252-24256.

Kusama T, Mukai M, Ayaki M, Imamura F, Tatsuta M, Matsumoto Y, Nakamura H and Inoue M (2003) Inhibition of lysophosphatidic acid-induced RhoA activation and tumor cell invasion by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors. *Int J Oncol* **23:**1173-1178.

Lai YJ, Chen CS, Lin WC and Lin FT (2005) c-Src-mediated phosphorylation of TRIP6 regulates its function in lysophosphatidic acid-induced cell migration. *Mol Cell Biol* **25:**5859-5868.

Lai YJ, Lin WC and Lin FT (2007) PTPL1/FAP-1 negatively regulates TRIP6 function in lysophosphatidic acid-induced cell migration. *J Biol Chem* **282:**24381-24387.

LaPorte SL, Juo ZS, Vaclavikova J, Colf LA, Qi X, Heller NM, Keegan AD and Garcia KC (2008) Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell* **132:**259-272.

Lee CW, Rivera R, Dubin AE and Chun J (2007) LPA(4)/GPR23 is a lysophosphatidic acid (LPA) receptor utilizing G(s)-, G(q)/G(i)-mediated calcium signaling and G(12/13)-mediated Rho activation. *J Biol Chem* **282:**4310-4317.

Lee CW, Rivera R, Gardell S, Dubin AE and Chun J (2006) GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J Biol Chem* **281:**23589-23597.

Lee H, Goetzl EJ and An S (2000) Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am J Physiol Cell Physiol* **278:**C612-8.

Lee HJ and Zheng JJ (2010) PDZ domains and their binding partners: structure, specificity, and modification. *Cell Commun Signal* **8:**8-811X-8-8.

Lee M, Choi S, Hallden G, Yo SJ, Schichnes D and Aponte GW (2009) P2Y5 is a G{alpha}i, G{alpha}12/13 G Protein Coupled Receptor Activated by Lysophosphatidic Acid that Reduces Intestinal Cell Adhesion. *Am J Physiol Gastrointest Liver Physiol*

Lefkowitz RJ, Hausdorff WP and Caron MG (1990) Role of phosphorylation in desensitization of the beta-adrenoceptor. *Trends Pharmacol Sci* **11:**190-194.

Li H, Wang D, Zhang H, Kirmani K, Zhao Z, Steinmetz R and Xu Y (2009) Lysophosphatidic acid stimulates cell migration, invasion, and colony formation as well as tumorigenesis/metastasis of mouse ovarian cancer in immunocompetent mice. *Mol Cancer Ther* **8:**1692-1701.

Li Q, Schachter JB, Harden TK and Nicholas RA (1997) The 6H1 orphan receptor, claimed to be the p2y5 receptor, does not mediate nucleotide-promoted second messenger responses. *Biochem Biophys Res Commun* **236:**455-460.

Li S and Zhang J (2009) Lipopolysaccharide induces autotaxin expression in human monocytic THP-1 cells. *Biochem Biophys Res Commun* **378:**264-268.

Liliom K, Bittman R, Swords B and Tigyi G (1996) N-palmitoyl-serine and N-palmitoyl-tyrosine phosphoric acids are selective competitive antagonists of the lysophosphatidic acid receptors. *Mol Pharmacol* **50:**616-623.

Liliom K, Tsukahara T, Tsukahara R, Zelman-Femiak M, Swiezewska E, Tigyi G (2006) Farnesyl phosphates are endogenous ligands of lysophosphatidic acid receptors: inhibition of LPA GPCR and activation of PPARs. *Biochim Biophys Acta* **1761**:1506-1514.

Lin DA and Boyce JA (2005) IL-4 regulates MEK expression required for lysophosphatidic acid-mediated chemokine generation by human mast cells. *J Immunol* **175:**5430-5438.

Lin FT and Lai YJ (2008) Regulation of the LPA2 receptor signaling through the carboxyl-terminal tail-mediated protein-protein interactions. *Biochim Biophys Acta* **1781:**558-562.

Lind SE, Smith DB, Janmey PA and Stossel TP (1988) Depression of gelsolin levels and detection of gelsolin-actin complexes in plasma of patients with acute lung injury. *Am Rev Respir Dis* **138:**429-434.

Lipp P and Reither G (2011) Protein kinase C: the "masters" of calcium and lipid. *Cold Spring Harb Perspect Biol* **3:**10.1101/cshperspect.a004556.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25:**402-408.

Logothetis DE, Kurachi Y, Galper J, Neer EJ and Clapham DE (1987) The beta gamma subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. *Nature* **325:**321-326.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG and Lefkowitz RJ (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283:**655-661.

Luttrell LM and Lefkowitz RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* **115:**455-465.

Lynch KR, Hopper DW, Carlisle SJ, Catalano JG, Zhang M and MacDonald TL (1997) Structure/activity relationships in lysophosphatidic acid: the 2-hydroxyl moiety. *Mol Pharmacol* **52:**75-81.

MacEwan DJ, Kim GD and Milligan G (1996) Agonist regulation of adenylate cyclase activity in neuroblastoma x glioma hybrid NG108-15 cells transfected to co-express adenylate cyclase type II and the beta 2-adrenoceptor. Evidence that adenylate cyclase is the limiting component for receptor-mediated stimulation of adenylate cyclase activity. *Biochem J* **318 (Pt 3):**1033-1039.

Madison JM (2003) Migration of airway smooth muscle cells. *Am J Respir Cell Mol Biol* **29:**8-11.

Mahon MJ, Donowitz M, Yun CC and Segre GV (2002) Na(+)/H(+) exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. *Nature* **417:**858-861.

Manning CD, Burman M, Christensen SB, Cieslinski LB, Essayan DM, Grous M, Torphy TJ and Barnette MS (1999) Suppression of human inflammatory cell function by subtype-selective PDE4 inhibitors correlates with inhibition of PDE4A and PDE4B. *Br J Pharmacol* **128:**1393-1398.

Martin AC, Willoughby D, Ciruela A, Ayling LJ, Pagano M, Wachten S, Tengholm A and Cooper DM (2009) Capacitative Ca2+ entry via Orai1 and stromal interacting molecule 1 (STIM1) regulates adenylyl cyclase type 8. *Mol Pharmacol* **75:**830-842.

Matas-Rico E, Garcia-Diaz B, Llebrez-Zayas P, Lopez-Barroso D, Santin L, Pedraza C, Smith-Fernandez A, Fernandez-Llebrez P, Tellez T, Redondo M, Chun J, De Fonseca FR and Estivill-Torrus G (2008) Deletion of lysophosphatidic acid receptor LPA1 reduces neurogenesis in the mouse dentate gyrus. *Mol Cell Neurosci* **39:**342-355.

Meerschaert K, De Corte V, De Ville Y, Vandekerckhove J and Gettemans J (1998) Gelsolin and functionally similar actin-binding proteins are regulated by lysophosphatidic acid. *EMBO J* **17:**5923-5932.

Mehats C, Jin SL, Wahlstrom J, Law E, Umetsu DT and Conti M (2003) PDE4D plays a critical role in the control of airway smooth muscle contraction. *FASEB J* **17:**1831-1841.

Meyer BH, Freuler F, Guerini D and Siehler S (2008) Reversible translocation of p115-RhoGEF by G(12/13)-coupled receptors. *J Cell Biochem* **104:**1660-1670.

Miles EW, Rhee S and Davies DR (1999) The molecular basis of substrate channeling. *J Biol Chem* **274:**12193-12196.

Millar JK, Pickard BS, Mackie S, James R, Christie S, Buchanan SR, Malloy MP, Chubb JE, Huston E, Baillie GS, Thomson PA, Hill EV, Brandon NJ, Rain JC, Camargo LM, Whiting PJ, Houslay MD, Blackwood DH, Muir WJ and Porteous DJ (2005) DISC1 and PDE4B are interacting genetic factors in schizophrenia that regulate cAMP signaling. *Science* **310:**1187-1191.

Milligan G (2003) Principles: extending the utility of [35S]GTP gamma S binding assays. *Trends Pharmacol Sci* **24:**87-90.

Milligan G and Kostenis E (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol* **147 Suppl 1:**S46-55.

Milligan G and Kostenis E (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol* **147 Suppl 1:**S46-55.

Mills GB and Moolenaar WH (2003) The emerging role of lysophosphatidic acid in cancer. *Nat Rev Cancer* **3:**582-591.

Mintzer E, Sargsyan H and Bittman R (2006) Lysophosphatidic acid and lipopolysaccharide bind to the PIP2-binding domain of gelsolin. *Biochim Biophys Acta* **1758:**85-89.

Mio T, Liu X, Toews ML and Rennard SI (2002) Lysophosphatidic acid augments fibroblast-mediated contraction of released collagen gels. *J Lab Clin Med* **139:**20-27.

Mistry R, Dowling MR and Challiss RA (2011) (3)(5)S]GTPgammaS binding as an index of total G-protein and Galpha-subtype-specific activation by GPCRs. *Methods Mol Biol* **746:**263-275.

Moolenaar WH and Perrakis A (2011) Insights into autotaxin: how to produce and present a lipid mediator. *Nat Rev Mol Cell Biol* **12:**674-679.

Mori M and Tsushima H (2000) Activation of Rho signaling contributes to lysophosphatidic acid-induced contraction of intact ileal smooth muscle of guinea-pig. *Can J Physiol Pharmacol* **78:**729-736.

Morris KE, Schang LM and Brindley DN (2006) Lipid phosphate phosphatase-2 activity regulates S-phase entry of the cell cycle in Rat2 fibroblasts. *J Biol Chem* **281:**9297-9306.

Moughal NA, Waters CM, Valentine WJ, Connell M, Richardson JC, Tigyi G, Pyne S and Pyne NJ (2006) Protean agonism of the lysophosphatidic acid receptor-1 with Ki16425 reduces nerve growth factor-induced neurite outgrowth in pheochromocytoma 12 cells. *J Neurochem* **98:**1920-1929.

Mu H, Calderone TL, Davies MA, Prieto VG, Wang H, Mills GB, Bar-Eli M and Gershenwald JE (2012) Lysophosphatidic acid induces lymphangiogenesis and IL-8 production in vitro in human lymphatic endothelial cells. *Am J Pathol* **180:**2170-2181.

Mukai M, Nakamura H, Tatsuta M, Iwasaki T, Togawa A, Imamura F and Akedo H (2000) Hepatoma cell migration through a mesothelial cell monolayer is inhibited by cyclic AMP-elevating agents via a Rho-dependent pathway. *FEBS Lett* **484:**69-73.

Mukhina S, Stepanova V, Traktouev D, Poliakov A, Beabealashvilly R, Gursky Y, Minashkin M, Shevelev A and Tkachuk V (2000) The chemotactic action of urokinase on smooth muscle cells is dependent on its kringle domain. Characterization of interactions and contribution to chemotaxis. *J Biol Chem* **275:**16450-16458.

Murakami M, Shiraishi A, Tabata K and Fujita N (2008) Identification of the orphan GPCR, P2Y(10) receptor as the sphingosine-1-phosphate and lysophosphatidic acid receptor. *Biochem Biophys Res Commun* **371:**707-712.

Murphy K (2012) *Janeway's immunobiology*. Garland Science; Taylor & Francis distributor, London; London.

Nakasaki T, Tanaka T, Okudaira S, Hirosawa M, Umemoto E, Otani K, Jin S, Bai Z, Hayasaka H, Fukui Y, Aozasa K, Fujita N, Tsuruo T, Ozono K, Aoki J and Miyasaka M (2008) Involvement of the lysophosphatidic acid-generating enzyme autotaxin in lymphocyte-endothelial cell interactions. *Am J Pathol* **173:**1566-1576.

Nakata J, Kondo M, Tamaoki J, Takemiya T, Nohara M, Yamagata K and Nagai A (2005) Augmentation of allergic inflammation in the airways of cyclooxygenase-2-deficient mice. *Respirology* **10:**149-156.

Newby AC and Zaltsman AB (2000) Molecular mechanisms in intimal hyperplasia. *J Pathol* **190:**300-309.

Nguyen C, Teo JL, Matsuda A, Eguchi M, Chi EY, Henderson WR,Jr and Kahn M (2003) Chemogenomic identification of Ref-1/AP-1 as a therapeutic target for asthma. *Proc Natl Acad Sci U S A* **100:**1169-1173.

Niimi K, Ge Q, Moir LM, Ammit AJ, Trian T, Burgess JK, Black JL and Oliver BG (2012) beta2-Agonists upregulate PDE4 mRNA but not protein or activity in human airway smooth muscle cells from asthmatic and nonasthmatic volunteers. *Am J Physiol Lung Cell Mol Physiol* **302:**L334-42.

Nikitopoulou I, Oikonomou N, Karouzakis E, Sevastou I, Nikolaidou-Katsaridou N, Zhao Z, Mersinias V, Armaka M, Xu Y, Masu M, Mills GB, Gay S, Kollias G and Aidinis V (2012) Autotaxin expression from synovial fibroblasts is essential for the pathogenesis of modeled arthritis. *J Exp Med* **209:**925-933.

Nino G, Hu A, Grunstein JS and Grunstein MM (2009) Mechanism regulating proasthmatic effects of prolonged homologous beta2-adrenergic receptor desensitization in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* **297:**L746-57.

Nishimasu H, Ishitani R, Aoki J and Nureki O (2012) A 3D view of autotaxin. *Trends Pharmacol Sci* **33:**138-145.

Nishimasu H, Okudaira S, Hama K, Mihara E, Dohmae N, Inoue A, Ishitani R, Takagi J, Aoki J and Nureki O (2011) Crystal structure of autotaxin and insight into GPCR activation by lipid mediators. *Nat Struct Mol Biol* **18:**205-212.

Nochi H, Tomura H, Tobo M, Tanaka N, Sato K, Shinozaki T, Kobayashi T, Takagishi K, Ohta H, Okajima F and Tamoto K (2008) Stimulatory role of lysophosphatidic acid in cyclooxygenase-2 induction by synovial fluid of patients with rheumatoid arthritis in fibroblast-like synovial cells. *J Immunol* **181:**5111-5119.

Nogami M, Whittle SM, Romberger DJ, Rennard SI and Toews ML (1995) Lysophosphatidic acid regulation of cyclic AMP accumulation in cultured human airway smooth muscle cells. *Mol Pharmacol* **48:**766-773.

Nogami M, Whittle SM, Romberger DJ, Rennard SI and Toews ML (1995) Lysophosphatidic acid regulation of cyclic AMP accumulation in cultured human airway smooth muscle cells. *Mol Pharmacol* **48:**766-773.

Noguchi K, Ishii S and Shimizu T (2003) Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J Biol Chem* **278:**25600-25606.

Noguchi Y, Okamoto A, Kasama T, Imajoh-Ohmi S, Karatsu T and Nogawa H (2006) Lysophosphatidic acid cooperates with EGF in inducing branching morphogenesis of embryonic mouse salivary epithelium. *Dev Dyn* **235:**403-410.

Northup JK, Sternweis PC, Smigel MD, Schleifer LS, Ross EM and Gilman AG (1980) Purification of the regulatory component of adenylate cyclase. *Proc Natl Acad Sci U S A* **77:**6516-6520.

Offermanns S and Simon MI (1995) G alpha 15 and G alpha 16 couple a wide variety of receptors to phospholipase C. *J Biol Chem* **270:**15175-15180.

Oguma T, Asano K, Shiomi T, Fukunaga K, Suzuki Y, Nakamura M, Matsubara H, Sheldon HK, Haley KJ, Lilly CM, Drazen JM and Yamaguchi K (2002) Cyclooxygenase-2 expression during allergic inflammation in guinea-pig lungs. *Am J Respir Crit Care Med* **165:**382-386.

Oguma T, Kume H, Ito S, Takeda N, Honjo H, Kodama I, Shimokata K and Kamiya K (2006) Involvement of reduced sensitivity to Ca in beta-adrenergic action on airway smooth muscle. *Clin Exp Allergy* **36:**183-191.

Oh DY, Yoon JM, Moon MJ, Hwang JI, Choe H, Lee JY, Kim JI, Kim S, Rhim H, O'Dell DK, Walker JM, Na HS, Lee MG, Kwon HB, Kim K and Seong JY (2008) Identification of farnesyl pyrophosphate and N-arachidonylglycine as endogenous ligands for GPR92. *J Biol Chem* **283:**21054-21064.

Oh YS, Jo NW, Choi JW, Kim HS, Seo SW, Kang KO, Hwang JI, Heo K, Kim SH, Kim YH, Kim IH, Kim JH, Banno Y, Ryu SH and Suh PG (2004) NHERF2 specifically interacts with LPA2 receptor and defines the specificity and efficiency of receptor-mediated phospholipase C-beta3 activation. *Mol Cell Biol* **24:**5069-5079.

Ohata H, Aizawa H and Momose K (1997) Lysophosphatidic acid sensitizes mechanical stress-induced Ca2+ response via activation of phospholipase C and tyrosine kinase in cultured smooth muscle cells. *Life Sci* **60:**1287-1295.

Ohta H, Sato K, Murata N, Damirin A, Malchinkhuu E, Kon J, Kimura T, Tobo M, Yamazaki Y, Watanabe T, Yagi M, Sato M, Suzuki R, Murooka H, Sakai T, Nishitoba T, Im DS, Nochi H, Tamoto K, Tomura H and Okajima F (2003) Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. *Mol Pharmacol* **64:**994-1005.

Ohuchi H, Hamada A, Matsuda H, Takagi A, Tanaka M, Aoki J, Arai H and Noji S (2008) Expression patterns of the lysophospholipid receptor genes during mouse early development. *Dev Dyn* **237:**3280-3294.

Oikonomou N, Mouratis MA, Tzouvelekis A, Kaffe E, Valavanis C, Vilaras G, Karameris A, Prestwich GD, Bouros D and Aidinis V (2012) Pulmonary autotaxin expression contributes to the pathogenesis of pulmonary fibrosis. *Am J Respir Cell Mol Biol* **47:**566-574.

Oka S, Ota R, Shima M, Yamashita A, Sugiura T (2010) GPR35 is a novel lysophosphatidic acid receptor. *Biochem Biophys Res Commun* **395**:232-237.

Okumura M, Kato K, Fukui R, Fukushima N and Tsujiuchi T (2011) No Involvement of Lysophosphatidic Acid Receptor-3 in Cell Migration of Mouse Lung Tumor Cells Stimulatedby 12-O-Tetradecanoylphorbol-13-acetate. *J Toxicol Pathol* **24:**183-186.

Okusa MD, Ye H, Huang L, Sigismund L, Macdonald T and Lynch KR (2003) Selective blockade of lysophosphatidic acid LPA3 receptors reduces murine renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol* **285:**F565-74.

Osborn TM, Dahlgren C, Hartwig JH and Stossel TP (2007) Modifications of cellular responses to lysophosphatidic acid and platelet-activating factor by plasma gelsolin. *Am J Physiol Cell Physiol* **292:**C1323-30.

Osborn TM, Verdrengh M, Stossel TP, Tarkowski A and Bokarewa M (2008) Decreased levels of the gelsolin plasma isoform in patients with rheumatoid arthritis. *Arthritis Res Ther* **10:**R117.

Oyesanya RA, Lee ZP, Wu J, Chen J, Song Y, Mukherjee A, Dent P, Kordula T, Zhou H and Fang X (2008) Transcriptional and post-transcriptional mechanisms for lysophosphatidic acid-induced cyclooxygenase-2 expression in ovarian cancer cells. *FASEB J* **22:**2639-2651.

Page CP and Spina D (2012) Selective PDE inhibitors as novel treatments for respiratory diseases. *Curr Opin Pharmacol* **12:**275-286.

Pamuklar Z, Federico L, Liu S, Umezu-Goto M, Dong A, Panchatcharam M, Fulkerson Z, Berdyshev E, Natarajan V, Fang X, van Meeteren LA, Moolenaar WH, Mills GB, Morris AJ and Smyth SS (2009) Autotaxin/lysopholipase D and lysophosphatidic acid regulate murine hemostasis and thrombosis. *J Biol Chem* **284:**7385-7394.

Panchatcharam M, Miriyala S, Yang F, Rojas M, End C, Vallant C, Dong A, Lynch K, Chun J, Morris AJ and Smyth SS (2008) Lysophosphatidic acid receptors 1 and 2 play roles in regulation of vascular injury responses but not blood pressure. *Circ Res* **103:**662-670.

Panetti TS, Nowlen J and Mosher DF (2000) Sphingosine-1-phosphate and lysophosphatidic acid stimulate endothelial cell migration. *Arterioscler Thromb Vasc Biol* **20:**1013-1019.

Paredes RM, Etzler JC, Watts LT, Zheng W and Lechleiter JD (2008) Chemical calcium indicators. *Methods* **46:**143-151.

Park GY, Lee YG, Berdyshev E, Nyenhuis S, Du J, Fu P, Gorshkova IA, Li Y, Chung S, Karpurapu M, Deng J, Ranjan R, Xiao L, Jaffe HA, Corbridge SJ, Kelly EA, Jarjour NN, Chun J, Prestwich GD, Kaffe E, Ninou I, Aidinis V, Morris AJ, Smyth SS, Ackerman SJ, Natarajan V and Christman JW (2013) Autotaxinproduction of Lysophosphatidic Acid Mediates Allergic Asthmatic Inflammation. *Am J Respir Crit Care Med*

Pasternack SM, von Kugelgen I, Aboud KA, Lee YA, Ruschendorf F, Voss K, Hillmer AM, Molderings GJ, Franz T, Ramirez A, Nurnberg P, Nothen MM and Betz RC

(2008) G protein-coupled receptor P2Y5 and its ligand LPA are involved in maintenance of human hair growth. *Nat Genet* **40:**329-334.

Pease JE and Sabroe I (2002) The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy. *Am J Respir Med* **1:**19-25.

Pegorier S, Arouche N, Dombret MC, Aubier M and Pretolani M (2007) Augmented epithelial endothelin-1 expression in refractory asthma. *J Allergy Clin Immunol* **120:**1301-1307.

Penn RB, Panettieri RA,Jr and Benovic JL (1998) Mechanisms of acute desensitization of the beta2AR-adenylyl cyclase pathway in human airway smooth muscle. *Am J Respir Cell Mol Biol* **19:**338-348.

Pertz O, Hodgson L, Klemke RL and Hahn KM (2006) Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature* **440:**1069-1072.

Pierce KL and Lefkowitz RJ (2001) Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat Rev Neurosci* **2:**727-733.

Pierce KL, Premont RT and Lefkowitz RJ (2002) Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* **3:**639-650.

Pilpel Y and Segal M (2006) The role of LPA1 in formation of synapses among cultured hippocampal neurons. *J Neurochem* **97:**1379-1392.

Pilquil C, Singh I, Zhang QX, Ling ZC, Buri K, Stromberg LM, Dewald J and Brindley DN (2001) Lipid phosphate phosphatase-1 dephosphorylates exogenous lysophosphatidate and thereby attenuates its effects on cell signalling. *Prostaglandins Other Lipid Mediat* **64:**83-92.

Pradere JP, Gonzalez J, Klein J, Valet P, Gres S, Salant D, Bascands JL, Saulnier-Blache JS and Schanstra JP (2008) Lysophosphatidic acid and renal fibrosis. *Biochim Biophys Acta* **1781:**582-587.

Pradere JP, Tarnus E, Gres S, Valet P and Saulnier-Blache JS (2007) Secretion and lysophospholipase D activity of autotaxin by adipocytes are controlled by N-glycosylation and signal peptidase. *Biochim Biophys Acta* **1771:**93-102.

Prescott SL, Macaubas C, Holt BJ, Smallacombe TB, Loh R, Sly PD and Holt PG (1998) Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol* **160:**4730-4737.

Prestwich GD, Gajewiak J, Zhang H, Xu X, Yang G and Serban M (2008) Phosphatase-resistant analogues of lysophosphatidic acid: agonists promote healing, antagonists and autotaxin inhibitors treat cancer. *Biochim Biophys Acta* **1781:**588-594.

Radhika V, Hee Ha J, Jayaraman M, Tsim ST and Dhanasekaran N (2005) Mitogenic signaling by lysophosphatidic acid (LPA) involves Galpha12. *Oncogene* **24:**4597-4603.

Rang HP, Dale MM, Ritter JM and Moore PK (2003) *Pharmacology.* Churchill Livingstone, Edinburgh.

Raval CM and Lee PJ (2010) Heme oxygenase-1 in lung disease. *Curr Drug Targets* **11:**1532-1540.

Regamey N, Ochs M, Hilliard TN, Muhlfeld C, Cornish N, Fleming L, Saglani S, Alton EW, Bush A, Jeffery PK and Davies JC (2008) Increased airway smooth muscle mass in children with asthma, cystic fibrosis, and non-cystic fibrosis bronchiectasis. *Am J Respir Crit Care Med* **177:**837-843.

Ren J, Xiao YJ, Singh LS, Zhao X, Zhao Z, Feng L, Rose TM, Prestwich GD and Xu Y (2006) Lysophosphatidic acid is constitutively produced by human peritoneal mesothelial cells and enhances adhesion, migration, and invasion of ovarian cancer cells. *Cancer Res* **66:**3006-3014.

Ren XD and Schwartz MA (2000) Determination of GTP loading on Rho. *Methods Enzymol* **325:**264-272.

Rinard GA, Rubinfeld AR, Brunton LL and Mayer SE (1979) Depressed cyclic AMP levels in airways smooth muscle from asthmatic dogs. *Proc Natl Acad Sci U S A* **76:**1472-1476.

Riobo NA and Manning DR (2005) Receptors coupled to heterotrimeric G proteins of the G12 family. *Trends Pharmacol Sci* **26:**146-154.

Rivera-Lopez CM, Tucker AL and Lynch KR (2008) Lysophosphatidic acid (LPA) and angiogenesis. *Angiogenesis* **11:**301-310.

Roberts C, Winter P, Shilliam CS, Hughes ZA, Langmead C, Maycox PR and Dawson LA (2005) Neurochemical changes in LPA1 receptor deficient mice--a putative model of schizophrenia. *Neurochem Res* **30:**371-377.

Roberts R, Sciorra VA and Morris AJ (1998) Human type 2 phosphatidic acid phosphohydrolases. Substrate specificity of the type 2a, 2b, and 2c enzymes and cell surface activity of the 2a isoform. *J Biol Chem* **273:**22059-22067.

Rodriguez Perez CE, Nie W, Sinnett-Smith J, Rozengurt E and Yoo J (2011) TNF-alpha potentiates lysophosphatidic acid-induced COX-2 expression via PKD in human colonic myofibroblasts. *Am J Physiol Gastrointest Liver Physiol* **300:**G637-46.

Rozen S and Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132:**365-386.

Rubenfeld J, Guo J, Sookrung N, Chen R, Chaicumpa W, Casolaro V, Zhao Y, Natarajan V and Georas S (2006) Lysophosphatidic acid enhances interleukin-13 gene expression and promoter activity in T cells. *Am J Physiol Lung Cell Mol Physiol* **290:**L66-74.

Saatian B, Zhao Y, He D, Georas SN, Watkins T, Spannhake EW and Natarajan V (2006) Transcriptional regulation of lysophosphatidic acid-induced interleukin-8 expression and secretion by p38 MAPK and JNK in human bronchial epithelial cells. *Biochem J* **393:**657-668.

Sakai J, Oike M, Hirakawa M and Ito Y (2003) Theophylline and cAMP inhibit lysophosphatidic acid-induced hyperresponsiveness of bovine tracheal smooth muscle cells. *J Physiol* **549:**171-180.

Sakai J, Oike M, Hirakawa M and Ito Y (2003) Theophylline and cAMP inhibit lysophosphatidic acid-induced hyperresponsiveness of bovine tracheal smooth muscle cells. *J Physiol* **549:**171-180.

Sakai T, de la Pena JM and Mosher DF (1999) Synergism among lysophosphatidic acid, beta1A integrins, and epidermal growth factor or platelet-derived growth factor in mediation of cell migration. *J Biol Chem* **274:**15480-15486.

Samadi N, Bekele R, Capatos D, Venkatraman G, Sariahmetoglu M and Brindley DN (2011) Regulation of lysophosphatidate signaling by autotaxin and lipid phosphate phosphatases with respect to tumor progression, angiogenesis, metastasis and chemo-resistance. *Biochimie* **93:**61-70.

Santos WL, Rossi JA, Boggs SD and MacDonald TL (2000) The molecular pharmacology of lysophosphatidate signaling. *Ann N Y Acad Sci* **905:**232-241.

Sato K, Horiuchi Y, Jin Y, Malchinkhuu E, Komachi M, Kondo T and Okajima F (2011) Unmasking of LPA(1) receptor-mediated migration response to lysophosphatidic acid by interleukin-1beta-induced attenuation of Rho signaling pathways in rat astrocytes. *J Neurochem*

Schober A and Siess W (2012) Lysophosphatidic acid in atherosclerotic diseases. *Br J Pharmacol* **167:**465-482.

Sedlakova I, Vavrova J, Tosner J and Hanousek L (2008) Lysophosphatidic acid: an ovarian cancer marker. *Eur J Gynaecol Oncol* **29:**511-514.

Seewald S, Sachinidis A, Dusing R, Ko Y, Seul C, Epping P and Vetter H (1997) Lysophosphatidic acid and intracellular signalling in vascular smooth muscle cells. *Atherosclerosis* **130:**121-131.

Seewald S, Schmitz U, Seul C, Ko Y, Sachinidis A and Vetter H (1999) Lysophosphatidic acid stimulates protein kinase C isoforms alpha, beta, epsilon, and zeta in a pertussis toxin sensitive pathway in vascular smooth muscle cells. *Am J Hypertens* **12:**532-537.

Sevastou I, Kaffe E, Mouratis MA and Aidinis V (2013) Lysoglycerophospholipids in chronic inflammatory disorders: the PLA(2)/LPC and ATX/LPA axes. *Biochim Biophys Acta* **1831:**42-60.

Sexton PM, Morfis M, Tilakaratne N, Hay DL, Udawela M, Christopoulos G and Christopoulos A (2006) Complexing receptor pharmacology: modulation of family B G protein-coupled receptor function by RAMPs. *Ann N Y Acad Sci* **1070:**90-104.

Shano S, Moriyama R, Chun J and Fukushima N (2008) Lysophosphatidic acid stimulates astrocyte proliferation through LPA1. *Neurochem Int* **52:**216-220.

Shen Y, Rampino MA, Carroll RC and Nawy S (2012) G-protein-mediated inhibition of the Trp channel TRPM1 requires the Gbetagamma dimer. *Proc Natl Acad Sci U S A* **109:**8752-8757.

Shimada H and Rajagopalan LE (2010) Rho-kinase mediates lysophosphatidic acid-induced IL-8 and MCP-1 production via p38 and JNK pathways in human endothelial cells. *FEBS Lett* **584:**2827-2832.

Shimizu H, Daly JW and Creveling CR (1969) A radioisotopic method for measuring the formation of adenosine 3',5'-cyclic monophosphate in incubated slices of brain. *J Neurochem* **16:**1609-1619.

Shimomura Y (2012) Congenital hair loss disorders: rare, but not too rare. *J Dermatol* **39:**3-10.

Shin YS, Takeda K, Shiraishi Y, Jia Y, Wang M, Jackson L, Wright AD, Carter L, Robinson J, Hicken E and Gelfand EW (2012) Inhibition of Pim1 kinase activation attenuates allergen-induced airway hyperresponsiveness and inflammation. *Am J Respir Cell Mol Biol* **46:**488-497.

Shiomi T, Boudreault F, Padem N, Higashiyama S, Drazen JM and Tschumperlin DJ (2011) Lysophosphatidic acid stimulates epidermal growth factor-family ectodomain shedding and paracrine signaling from human lung fibroblasts. *Wound Repair Regen* **19:**229-240.

Siehler S (2009) Regulation of RhoGEF proteins by G12/13-coupled receptors. *Br J Pharmacol* **158:**41-49.

Siehler S (2008) Cell-based assays in GPCR drug discovery. *Biotechnol J* **3:**471-483.

Silacci P, Mazzolai L, Gauci C, Stergiopulos N, Yin HL and Hayoz D (2004) Gelsolin superfamily proteins: key regulators of cellular functions. *Cell Mol Life Sci* **61:**2614-2623.

Simon MF, Chap H and Douste-Blazy L (1982) Human platelet aggregation induced by 1-alkyl-lysophosphatidic acid and its analogs: a new group of phospholipid mediators? *Biochem Biophys Res Commun* **108:**1743-1750.

Skippen A, Swigart P and Cockcroft S (2013) Measurement of phospholipase C by monitoring inositol phosphates using [(3)H]inositol labeling protocols in permeabilized cells. *Methods Mol Biol* **937:**163-174.

Smith EP, Shanks K, Lipsky MM, DeTolla LJ, Keegan AD and Chapoval SP (2011) Expression of neuroimmune semaphorins 4A and 4D and their receptors in the lung is enhanced by allergen and vascular endothelial growth factor. *BMC Immunol* **12:**30.

Sondek J, Bohm A, Lambright DG, Hamm HE and Sigler PB (1996) Crystal structure of a G-protein beta gamma dimer at 2.1A resolution. *Nature* **379:**369-374.

Song HY, Lee MJ, Kim MY, Kim KH, Lee IH, Shin SH, Lee JS and Kim JH (2010) Lysophosphatidic acid mediates migration of human mesenchymal stem cells stimulated by synovial fluid of patients with rheumatoid arthritis. *Biochim Biophys Acta* **1801:**23-30.

Song J, Clair T, Noh JH, Eun JW, Ryu SY, Lee SN, Ahn YM, Kim SY, Lee SH, Park WS, Yoo NJ, Lee JY and Nam SW (2005) Autotaxin (lysoPLD/NPP2) protects fibroblasts from apoptosis through its enzymatic product, lysophosphatidic acid, utilizing albumin-bound substrate. *Biochem Biophys Res Commun* **337:**967-975.

Sonoda H, Aoki J, Hiramatsu T, Ishida M, Bandoh K, Nagai Y, Taguchi R, Inoue K and Arai H (2002) A novel phosphatidic acid-selective phospholipase A1 that produces lysophosphatidic acid. *J Biol Chem* **277:**34254-34263.

Stefan C, Jansen S and Bollen M (2005) NPP-type ectophosphodiesterases: unity in diversity. *Trends Biochem Sci* **30:**542-550.

Sterne-Marr R, Gurevich VV, Goldsmith P, Bodine RC, Sanders C, Donoso LA and Benovic JL (1993) Polypeptide variants of beta-arrestin and arrestin3. *J Biol Chem* **268:**15640-15648.

Stewart AG, Harris T, Fernandes DJ, Schachte LC, Koutsoubos V, Guida E, Ravenhall CE, Vadiveloo P and Wilson JW (1999) Beta2-adrenergic receptor agonists and cAMP arrest human cultured airway smooth muscle cells in the G(1) phase of the cell cycle: role of proteasome degradation of cyclin D1. *Mol Pharmacol* **56:**1079-1086.

Stracke ML, Krutzsch HC, Unsworth EJ, Arestad A, Cioce V, Schiffmann E and Liotta LA (1992) Identification, purification, and partial sequence analysis of autotaxin, a novel motility-stimulating protein. *J Biol Chem* **267:**2524-2529.

Strathmann MP and Simon MI (1991) G alpha 12 and G alpha 13 subunits define a fourth class of G protein alpha subunits. *Proc Natl Acad Sci U S A* **88:**5582-5586.

Sturm A, Sudermann T, Schulte KM, Goebell H and Dignass AU (1999) Modulation of intestinal epithelial wound healing in vitro and in vivo by lysophosphatidic acid. *Gastroenterology* **117:**368-377.

Sugiura T, Nakane S, Kishimoto S, Waku K, Yoshioka Y and Tokumura A (2002) Lysophosphatidic acid, a growth factor-like lipid, in the saliva. *J Lipid Res* **43:**2049-2055.

Sugiura T, Tokumura A, Gregory L, Nouchi T, Weintraub ST and Hanahan DJ (1994) Biochemical characterization of the interaction of lipid phosphoric acids with human platelets: comparison with platelet activating factor. *Arch Biochem Biophys* **311:**358-368.

Suhler E, Lin W, Yin HL and Lee WM (1997) Decreased plasma gelsolin concentrations in acute liver failure, myocardial infarction, septic shock, and myonecrosis. *Crit Care Med* **25:**594-598.

Sumida H, Noguchi K, Kihara Y, Abe M, Yanagida K, Hamano F, Sato S, Tamaki K, Morishita Y, Kano MR, Iwata C, Miyazono K, Sakimura K, Shimizu T and Ishii S (2010) LPA4 regulates blood and lymphatic vessel formation during mouse embryogenesis. *Blood* **116:**5060-5070.

Swaisgood CM, Aronica MA, Swaidani S and Plow EF (2007) Plasminogen is an important regulator in the pathogenesis of a murine model of asthma. *Am J Respir Crit Care Med* **176:**333-342.

Tabata K, Baba K, Shiraishi A, Ito M and Fujita N (2007) The orphan GPCR GPR87 was deorphanized and shown to be a lysophosphatidic acid receptor. *Biochem Biophys Res Commun* **363:**861-866.

Tabchy A, Tigyi G and Mills GB (2011) Location, location, location: a crystal-clear view of autotaxin saturating LPA receptors. *Nat Struct Mol Biol* **18:**117-118.

Tager AM, Kradin RL, LaCamera P, Bercury SD, Campanella GS, Leary CP, Polosukhin V, Zhao LH, Sakamoto H, Blackwell TS and Luster AD (2004) Inhibition of pulmonary fibrosis by the chemokine IP-10/CXCL10. *Am J Respir Cell Mol Biol* **31:**395-404.

Tager AM, LaCamera P, Shea BS, Campanella GS, Selman M, Zhao Z, Polosukhin V, Wain J, Karimi-Shah BA, Kim ND, Hart WK, Pardo A, Blackwell TS, Xu Y, Chun J and Luster AD (2008) The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat Med* **14:**45-54.

Tager AM, LaCamera P, Shea BS, Campanella GS, Selman M, Zhao Z, Polosukhin V, Wain J, Karimi-Shah BA, Kim ND, Hart WK, Pardo A, Blackwell TS, Xu Y, Chun J and Luster AD (2008) The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat Med* **14:**45-54.

Takasaki J, Saito T, Taniguchi M, Kawasaki T, Moritani Y, Hayashi K and Kobori M (2004) A novel Galphaq/11-selective inhibitor. *J Biol Chem* **279:**47438-47445.

Takeda N, Sumi Y, Prefontaine D, Al Abri J, Al Heialy N, Al-Ramli W, Michoud MC, Martin JG and Hamid Q (2009) Epithelium-derived chemokines induce airway smooth muscle cell migration. *Clin Exp Allergy* **39:**1018-1026.

Takuwa Y, Takuwa N and Sugimoto N (2002) The Edg family G protein-coupled receptors for lysophospholipids: their signaling properties and biological activities. *J Biochem* **131:**767-771.

Tanaka M, Okudaira S, Kishi Y, Ohkawa R, Iseki S, Ota M, Noji S, Yatomi Y, Aoki J and Arai H (2006) Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. *J Biol Chem* **281:**25822-25830.

Tang WJ and Gilman AG (1991) Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. *Science* **254:**1500-1503.

Taussig R, Tang WJ, Hepler JR and Gilman AG (1994) Distinct patterns of bidirectional regulation of mammalian adenylyl cyclases. *J Biol Chem* **269:**6093-6100.

Thellin O, ElMoualij B, Heinen E and Zorzi W (2009) A decade of improvements in quantification of gene expression and internal standard selection. *Biotechnol Adv* **27:**323-333.

Thompson BD, Jin Y, Wu KH, Colvin RA, Luster AD, Birnbaumer L and Wu MX (2007) Inhibition of G alpha i2 activation by G alpha i3 in CXCR3-mediated signaling. *J Biol Chem* **282:**9547-9555.

Thoreson WB, Ryan JS, Shi C, Kelly ME, Bryson EJ, Toews ML, Ediger TL and Chacko DM (2002) Lysophosphatidic acid receptor signaling in mammalian retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* **43:**2450-2461.

Thumser AE, Voysey JE and Wilton DC (1994) The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin. *Biochem J* **301 (Pt 3):**801-806.

Tigyi G, Dyer DL and Miledi R (1994) Lysophosphatidic acid possesses dual action in cell proliferation. *Proc Natl Acad Sci U S A* **91:**1908-1912.

Tigyi G, Fischer DJ, Sebok A, Marshall F, Dyer DL and Miledi R (1996) Lysophosphatidic acid-induced neurite retraction in PC12 cells: neurite-protective effects of cyclic AMP signaling. *J Neurochem* **66:**549-558.

Tigyi G, Fischer DJ, Sebok A, Yang C, Dyer DL and Miledi R (1996) Lysophosphatidic acid-induced neurite retraction in PC12 cells: control by phosphoinositide-Ca2+ signaling and Rho. *J Neurochem* **66:**537-548.

Tigyi G, Henschen A and Miledi R (1991) A factor that activates oscillatory chloride currents in Xenopus oocytes copurifies with a subfraction of serum albumin. *J Biol Chem* **266:**20602-20609.

Tigyi G and Miledi R (1992) Lysophosphatidates bound to serum albumin activate membrane currents in Xenopus oocytes and neurite retraction in PC12 pheochromocytoma cells. *J Biol Chem* **267:**21360-21367.

Tobin AB, Butcher AJ and Kong KC (2008) Location, location, location...site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends Pharmacol Sci* **29:**413-420.

Toews ML, Ustinova EE and Schultz HD (1997) Lysophosphatidic acid enhances contractility of isolated airway smooth muscle. *J Appl Physiol* **83:**1216-1222.

Toews ML, Ustinova EE and Schultz HD (1997) Lysophosphatidic acid enhances contractility of isolated airway smooth muscle. *J Appl Physiol* **83:**1216-1222.

Tokumura A (1995) A family of phospholipid autacoids: occurrence, metabolism and bioactions. *Prog Lipid Res* **34:**151-184.

Tokumura A, Fukuzawa K and Tsukatani H (1978) Effects of synthetic and natural lysophosphatidic acids on the arterial blood pressure of different animal species. *Lipids* **13:**572-574.

Tokumura A, Fukuzawa K, Yamada S and Tsukatani H (1980) Stimulatory effect of lysophosphatidic acids on uterine smooth muscles of non-pregant rats. *Arch Int Pharmacodyn Ther* **245:**74-83.

Tokumura A, Majima E, Kariya Y, Tominaga K, Kogure K, Yasuda K and Fukuzawa K (2002) Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. *J Biol Chem* **277:**39436-39442.

Tokumura A, Sinomiya J, Kishimoto S, Tanaka T, Kogure K, Sugiura T, Satouchi K, Waku K and Fukuzawa K (2002) Human platelets respond differentially to lysophosphatidic acids having a highly unsaturated fatty acyl group and alkyl ether-linked lysophosphatidic acids. *Biochem J* **365:**617-628.

Tokumura A, Yube N, Fujimoto H and Tsukatani H (1991) Lysophosphatidic acids induce contraction of rat isolated colon by two different mechanisms. *J Pharm Pharmacol* **43:**774-778.

Tomsig JL, Snyder AH, Berdyshev EV, Skobeleva A, Mataya C, Natarajan V, Brindley DN and Lynch KR (2009) Lipid phosphate phosphohydrolase type 1 (LPP1) degrades extracellular lysophosphatidic acid in vivo. *Biochem J* **419:**611-618.

Tou JS and Gill JS (2005) Lysophosphatidic acid increases phosphatidic acid formation, phospholipase D activity and degranulation by human neutrophils. *Cell Signal* **17:**77-82.

Trian T, Burgess JK, Niimi K, Moir LM, Ge Q, Berger P, Liggett SB, Black JL and Oliver BG (2011) beta2-Agonist induced cAMP is decreased in asthmatic airway smooth muscle due to increased PDE4D. *PLoS One* **6:**e20000.

Tsuda S, Okudaira S, Moriya-Ito K, Shimamoto C, Tanaka M, Aoki J, Arai H, Murakami-Murofushi K and Kobayashi T (2006) Cyclic phosphatidic acid is produced by autotaxin in blood. *J Biol Chem* **281:**26081-26088.

Umezu-Goto M, Kishi Y, Taira A, Hama K, Dohmae N, Takio K, Yamori T, Mills GB, Inoue K, Aoki J and Arai H (2002) Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J Cell Biol* **158:**227-233.

van Corven EJ, Groenink A, Jalink K, Eichholtz T and Moolenaar WH (1989) Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cell* **59:**45-54.

van Corven EJ, Hordijk PL, Medema RH, Bos JL and Moolenaar WH (1993) Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts. *Proc Natl Acad Sci U S A* **90:**1257-1261.

van der Bend RL, Brunner J, Jalink K, van Corven EJ, Moolenaar WH and van Blitterswijk WJ (1992) Identification of a putative membrane receptor for the bioactive phospholipid, lysophosphatidic acid. *EMBO J* **11:**2495-2501.

van Leeuwen FN, Giepmans BN, van Meeteren LA and Moolenaar WH (2003) Lysophosphatidic acid: mitogen and motility factor. *Biochem Soc Trans* **31:**1209-1212.

van Meeteren LA, Ruurs P, Stortelers C, Bouwman P, van Rooijen MA, Pradere JP, Pettit TR, Wakelam MJ, Saulnier-Blache JS, Mummery CL, Moolenaar WH and Jonkers J (2006) Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. *Mol Cell Biol* **26:**5015-5022.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by

geometric averaging of multiple internal control genes. *Genome Biol* **3:**RESEARCH0034.

Vignola AM, Chanez P, Campbell AM, Souques F, Lebel B, Enander I and Bousquet J (1998) Airway inflammation in mild intermittent and in persistent asthma. *Am J Respir Crit Care Med* **157:**403-409.

Virag T, Elrod DB, Liliom K, Sardar VM, Parrill AL, Yokoyama K, Durgam G, Deng W, Miller DD and Tigyi G (2003) Fatty alcohol phosphates are subtype-selective agonists and antagonists of lysophosphatidic acid receptors. *Mol Pharmacol* **63:**1032-1042.

Volckaert T, Dill E, Campbell A, Tiozzo C, Majka S, Bellusci S and De Langhe SP (2011) Parabronchial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. *J Clin Invest* **121:**4409-4419.

Waggoner DW, Xu J, Singh I, Jasinska R, Zhang QX and Brindley DN (1999) Structural organization of mammalian lipid phosphate phosphatases: implications for signal transduction. *Biochim Biophys Acta* **1439:**299-316.

Wang T, Dowal L, El-Maghrabi MR, Rebecchi M and Scarlata S (2000) The pleckstrin homology domain of phospholipase C-beta(2) links the binding of gbetagamma to activation of the catalytic core. *J Biol Chem* **275:**7466-7469.

Watanabe N, Ikeda H, Nakamura K, Ohkawa R, Kume Y, Aoki J, Hama K, Okudaira S, Tanaka M, Tomiya T, Yanase M, Tejima K, Nishikawa T, Arai M, Arai H, Omata M, Fujiwara K and Yatomi Y (2007) Both plasma lysophosphatidic acid and serum autotaxin levels are increased in chronic hepatitis C. *J Clin Gastroenterol* **41:**616-623.

Watson EL, Jacobson KL, Singh JC, Idzerda R, Ott SM, DiJulio DH, Wong ST and Storm DR (2000) The type 8 adenylyl cyclase is critical for Ca2+ stimulation of cAMP accumulation in mouse parotid acini. *J Biol Chem* **275:**14691-14699.

Watterson KR, Lanning DA, Diegelmann RF and Spiegel S (2007) Regulation of fibroblast functions by lysophospholipid mediators: potential roles in wound healing. *Wound Repair Regen* **15:**607-616.

Webb TE, Kaplan MG and Barnard EA (1996) Identification of 6H1 as a P2Y purinoceptor: P2Y5. *Biochem Biophys Res Commun* **219:**105-110.

Weiner JA and Chun J (1999) Schwann cell survival mediated by the signaling phospholipid lysophosphatidic acid. *Proc Natl Acad Sci U S A* **96:**5233-5238.

Weiner JA, Hecht JH and Chun J (1998) Lysophosphatidic acid receptor gene vzg-1/lpA1/edg-2 is expressed by mature oligodendrocytes during myelination in the postnatal murine brain. *J Comp Neurol* **398:**587-598.

Willars GB (2006) Mammalian RGS proteins: multifunctional regulators of cellular signalling. *Semin Cell Dev Biol* **17:**363-376.

Williams JR, Khandoga AL, Goyal P, Fells JI, Perygin DH, Siess W, Parrill AL, Tigyi G and Fujiwara Y (2009) Unique ligand selectivity of the GPR92/LPA5

lysophosphatidate receptor indicates role in human platelet activation. *J Biol Chem* **284:**17304-17319.

Willoughby D and Cooper DM (2007) Organization and Ca2+ regulation of adenylyl cyclases in cAMP microdomains. *Physiol Rev* **87:**965-1010.

Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL and Donaldson DD (1998) Interleukin-13: central mediator of allergic asthma. *Science* **282:**2258-2261.

Wing MR, Houston D, Kelley GG, Der CJ, Siderovski DP and Harden TK (2001) Activation of phospholipase C-epsilon by heterotrimeric G protein betagamma-subunits. *J Biol Chem* **276:**48257-48261.

Woclawek-Potocka I, Kondraciuk K and Skarzynski DJ (2009) Lysophosphatidic acid stimulates prostaglandin E2 production in cultured stromal endometrial cells through LPA1 receptor. *Exp Biol Med (Maywood)* **234:**986-993.

Xiao K, McClatchy DB, Shukla AK, Zhao Y, Chen M, Shenoy SK, Yates JR,3rd and Lefkowitz RJ (2007) Functional specialization of beta-arrestin interactions revealed by proteomic analysis. *Proc Natl Acad Sci U S A* **104:**12011-12016.

Xie W, Matsumoto M, Chun J and Ueda H (2008) Involvement of LPA1 receptor signaling in the reorganization of spinal input through Abeta-fibers in mice with partial sciatic nerve injury. *Mol Pain* **4:**46-8069-4-46.

Xu D, Isaacs C, Hall IP and Emala CW (2001) Human airway smooth muscle expresses 7 isoforms of adenylyl cyclase: a dominant role for isoform V. *Am J Physiol Lung Cell Mol Physiol* **281:**L832-43.

Xu Y, Gaudette DC, Boynton JD, Frankel A, Fang XJ, Sharma A, Hurteau J, Casey G, Goodbody A and Mellors A (1995) Characterization of an ovarian cancer activating factor in ascites from ovarian cancer patients. *Clin Cancer Res* **1:**1223-1232.

Yagami A, Orihara K, Morita H, Futamura K, Hashimoto N, Matsumoto K, Saito H and Matsuda A (2010) IL-33 mediates inflammatory responses in human lung tissue cells. *J Immunol* **185:**5743-5750.

Yamada T, Ohoka Y, Kogo M and Inagaki S (2005) Physical and functional interactions of the lysophosphatidic acid receptors with PDZ domain-containing Rho guanine nucleotide exchange factors (RhoGEFs). *J Biol Chem* **280:**19358-19363.

Yanagida K and Ishii S (2011) Non-Edg family LPA receptors: the cutting edge of LPA research. *J Biochem* **150**:223-232

Yanagida K, Ishii S, Hamano F, Noguchi K and Shimizu T (2007) LPA4/p2y9/GPR23 mediates rho-dependent morphological changes in a rat neuronal cell line. *J Biol Chem* **282:**5814-5824.

Yanagida K, Masago K, Nakanishi H, Kihara Y, Hamano F, Tajima Y, Taguchi R, Shimizu T and Ishii S (2009) Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA6. *J Biol Chem* **284:**17731-17741.

Yart A, Chap H and Raynal P (2002) Phosphoinositide 3-kinases in lysophosphatidic acid signaling: regulation and cross-talk with the Ras/mitogen-activated protein kinase pathway. *Biochim Biophys Acta* **1582:**107-111.

Ye X (2008) Lysophospholipid signaling in the function and pathology of the reproductive system. *Hum Reprod Update* **14:**519-536.

Ye X, Hama K, Contos JJ, Anliker B, Inoue A, Skinner MK, Suzuki H, Amano T, Kennedy G, Arai H, Aoki J and Chun J (2005) LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature* **435:**104-108.

Ye X, Ishii I, Kingsbury MA and Chun J (2002) Lysophosphatidic acid as a novel cell survival/apoptotic factor. *Biochim Biophys Acta* **1585:**108-113.

Ye X, Skinner MK, Kennedy G and Chun J (2008) Age-dependent loss of sperm production in mice via impaired lysophosphatidic acid signaling. *Biol Reprod* **79:**328-336.

Yoon CM, Hong BS, Moon HG, Lim S, Suh PG, Kim YK, Chae CB and Gho YS (2008) Sphingosine-1-phosphate promotes lymphangiogenesis by stimulating S1P1/Gi/PLC/Ca2+ signaling pathways. *Blood* **112:**1129-1138.

Yoshida K, Nishida W, Hayashi K, Ohkawa Y, Ogawa A, Aoki J, Arai H and Sobue K (2003) Vascular remodeling induced by naturally occurring unsaturated lysophosphatidic acid in vivo. *Circulation* **108:**1746-1752.

Yoshizaki H, Ohba Y, Kurokawa K, Itoh RE, Nakamura T, Mochizuki N, Nagashima K and Matsuda M (2003) Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. *J Cell Biol* **162:**223-232.

Yu H, Okada T, Kobayashi M, Abo-Elmatty DM, Jahangeer S and Nakamura S (2009) Roles of extracellular and intracellular sphingosine 1-phosphate in cell migration. *Genes Cells* **14:**597-605.

Yu S, Murph MM, Lu Y, Liu S, Hall HS, Liu J, Stephens C, Fang X and Mills GB (2008) Lysophosphatidic acid receptors determine tumorigenicity and aggressiveness of ovarian cancer cells. *J Natl Cancer Inst* **100:**1630-1642.

Yukiura H, Hama K, Nakanaga K, Tanaka M, Asaoka Y, Okudaira S, Arima N, Inoue A, Hashimoto T, Arai H, Kawahara A, Nishina H and Aoki J (2011) Autotaxin regulates vascular development via multiple lysophosphatidic acid (LPA) receptors in zebrafish. *J Biol Chem* **286:**43972-43983.

Zhang H, Wang D, Sun H, Hall RA and Yun CC (2007) MAGI-3 regulates LPA-induced activation of Erk and RhoA. *Cell Signal* **19:**261-268.

Zhang H, Xu X, Gajewiak J, Tsukahara R, Fujiwara Y, Liu J, Fells JI, Perygin D, Parrill AL, Tigyi G and Prestwich GD (2009) Dual activity lysophosphatidic acid receptor pan-antagonist/autotaxin inhibitor reduces breast cancer cell migration in vitro and causes tumor regression in vivo. *Cancer Res* **69:**5441-5449.

Zhang N, Sundberg JP and Gridley T (2000) Mice mutant for Ppap2c, a homolog of the germ cell migration regulator wunen, are viable and fertile. *Genesis* **27:**137-140.

Zhang Y, Chen YC, Krummel MF and Rosen SD (2012) Autotaxin through Lysophosphatidic Acid Stimulates Polarization, Motility, and Transendothelial Migration of Naive T Cells. *J Immunol*

Zhao C, Fernandes MJ, Prestwich GD, Turgeon M, Di Battista J, Clair T, Poubelle PE and Bourgoin SG (2008) Regulation of lysophosphatidic acid receptor expression and function in human synoviocytes: implications for rheumatoid arthritis? *Mol Pharmacol* **73:**587-600.

Zhao C, Sardella A, Chun J, Poubelle PE, Fernandes MJ and Bourgoin SG (2011) TNF-alpha promotes LPA1- and LPA3-mediated recruitment of leukocytes in vivo through CXCR2 ligand chemokines. *J Lipid Res* **52:**1307-1318.

Zhao J, Chen Q, Li H, Myerburg M, Spannhake EW, Natarajan V and Zhao Y (2012) Lysophosphatidic acid increases soluble ST2 expression in mouse lung and human bronchial epithelial cells. *Cell Signal* **24:**77-85.

Zhao J, He D, Berdyshev E, Zhong M, Salgia R, Morris AJ, Smyth SS, Natarajan V and Zhao Y (2011) Autotaxin induces lung epithelial cell migration through lysoPLD activity-dependent and -independent pathways. *Biochem J* **439:**45-55.

Zhao J, He D, Su Y, Berdyshev E, Chun J, Natarajan V and Zhao Y (2011) Lysophosphatidic acid receptor 1 modulates lipopolysaccharide-induced inflammation in alveolar epithelial cells and murine lungs. *Am J Physiol Lung Cell Mol Physiol* **301:**L547-56.

Zhao P and Abood ME (2013) GPR55 and GPR35 and their relationship to cannabinoid and lysophospholipid receptors. *Life Sci* **92:**453-457.

Zhao Y, He D, Saatian B, Watkins T, Spannhake EW, Pyne NJ and Natarajan V (2006) Regulation of lysophosphatidic acid-induced epidermal growth factor receptor transactivation and interleukin-8 secretion in human bronchial epithelial cells by protein kinase Cdelta, Lyn kinase, and matrix metalloproteinases. *J Biol Chem* **281:**19501-19511.

Zhao Y, He D, Zhao J, Wang L, Leff AR, Spannhake EW, Georas S and Natarajan V (2007) Lysophosphatidic acid induces interleukin-13 (IL-13) receptor alpha2 expression and inhibits IL-13 signaling in primary human bronchial epithelial cells. *J Biol Chem* **282:**10172-10179.

Zhao Y and Natarajan V (2009) Lysophosphatidic acid signaling in airway epithelium: role in airway inflammation and remodeling. *Cell Signal* **21:**367-377.

Zhao Y, Tong J, He D, Pendyala S, Evgeny B, Chun J, Sperling AI and Natarajan V (2009) Role of lysophosphatidic acid receptor LPA2 in the development of allergic airway inflammation in a murine model of asthma. *Respir Res* **10:**114-9921-10-114.

Zhao Y, Usatyuk PV, Cummings R, Saatian B, He D, Watkins T, Morris A, Spannhake EW, Brindley DN and Natarajan V (2005) Lipid phosphate phosphatase-1 regulates lysophosphatidic acid-induced calcium release, NF-kappaB activation and interleukin-8 secretion in human bronchial epithelial cells. *Biochem J* **385:**493-502.

Zheng T, Liu W, Oh SY, Zhu Z, Hu B, Homer RJ, Cohn L, Grusby MJ and Elias JA (2008) IL-13 receptor alpha2 selectively inhibits IL-13-induced responses in the murine lung. *J Immunol* **180:**522-529.

Zheng Y, Kong Y and Goetzl EJ (2001) Lysophosphatidic acid receptor-selective effects on Jurkat T cell migration through a Matrigel model basement membrane. *J Immunol* **166:**2317-2322.

Zheng Y, Voice JK, Kong Y and Goetzl EJ (2000) Altered expression and functional profile of lysophosphatidic acid receptors in mitogen-activated human blood T lymphocytes. *FASEB J* **14:**2387-2389.

Zhou A (2007) Functional structure of the somatomedin B domain of vitronectin. *Protein Sci* **16:**1502-1508.

Zhou D, Luini W, Bernasconi S, Diomede L, Salmona M, Mantovani A and Sozzani S (1995) Phosphatidic acid and lysophosphatidic acid induce haptotactic migration of human monocytes. *J Biol Chem* **270:**25549-25556.

Zhou WL, Sugioka M and Yamashita M (1999) Lysophosphatidic acid-induced Ca(2+) mobilization in the neural retina of chick embryo. *J Neurobiol* **41:**495-504.

Zhou Z, Subramanian P, Sevilmis G, Globke B, Soehnlein O, Karshovska E, Megens R, Heyll K, Chun J, Saulnier-Blache JS, Reinholz M, van Zandvoort M, Weber C and Schober A (2011) Lipoprotein-derived lysophosphatidic acid promotes atherosclerosis by releasing CXCL1 from the endothelium. *Cell Metab* **13:**592-600.

Zhou ZB, Niu JP and Zhang ZJ (2009) Receptor-mediated vascular smooth muscle migration induced by LPA involves p38 mitogen-activated protein kinase pathway activation. *Int J Mol Sci* **10:**3194-3208.

Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y and Elias JA (1999) Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* **103:**779-788.