Title: Endogenous LPA$_1$ receptor agonists demonstrate ligand bias between calcium and ERK signalling pathways in human lung fibroblasts.

Authors: Afrah Sattikar, Mark R Dowling, Elizabeth M Rosethorne

Institutes:

Novartis Institutes for Biomedical Research, Wimblehurst Road, Horsham, West Sussex. RH12 5AB, UK (AS, MRD, EMR)

Novartis Institutes for Biomedical Research, 250 Massachusetts Avenue, Cambridge, MA 02139 (MRD)

School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham. NG7 2UH (EMR)

Corresponding author:

Dr E M Rosethorne

School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham. NG7 2UH

Tel: +44 115 8230080

Email: elizabeth.rosethorne@nottingham.ac.uk

Abbreviations: AC, adenylyl cyclase; AM966, (4′-{4-[(R)-1-(2-chloro-phenyl)-ethoxycarbonylamino]-3-methyl-isoxazol-5-yl]-biphenyl-4-yl)-acetic acid; DPBS, Dulbecco's phosphate buffered saline solution; ERK, extracellular signal-regulated kinase; FBS, foetal bovine serum; GPCRs, G protein-coupled receptors; HBSS, Hanks' balanced salt solution; HLF, human lung fibroblasts; HPRT1, hypoxanthine phosphoribosyltransferase 1; LPA, 1-acyl-2-hydroxy-*sn*-glycero-3-phosphate; MTA, Multi-Target Analysis; pERK, ERK1/2 phosphorylation; PLC, phospholipase C; PTx, pertussis toxin; SRE, serum-response element; TBS, Tris Buffered Saline; TBS-T, TBS-tween;

Abstract

Background and Purpose Human lung fibroblasts (HLF) express high levels of the LPA_1 receptor, a G protein-coupled receptor (GPCR) that responds to the endogenous lipid mediator lysophosphatidic acid (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate; LPA). Several molecular species or analogues of LPA exist and have been detected in biological fluids such as serum and plasma. The most widely expressed of the LPA receptor family is the LPA_1 receptor, which predominantly couples to $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$ proteins. This promiscuity of coupling raises the possibility that some of the LPA analogues may bias the LPA_1 receptor towards one signalling pathway over another.

Experimental Approach Here we have explored the signalling profiles of a range of LPA analogues in HLF that endogenously express the LPA_1 receptor. HLF were treated with LPA analogues, and receptor activation monitored via calcium mobilization and extracellular signal-regulated kinase (ERK) phosphorylation.

Key Results These analyses demonstrated that 16:0, 17:0, 18:2 and C18:1 LPA analogues appear to exhibit ligand bias between ERK phosphorylation and calcium mobilization when compared to 18:1 LPA, one of the most abundant forms of LPA that has been found in human plasma.

Conclusion and Implications The importance of LPA as a key signalling molecule is apparent due to its widespread occurrence in biological fluids and its association with disease conditions such as fibrosis and cancer. These findings have important, as yet unexplored, implications for the (patho) physiological signalling of the LPA_1 receptor, as it may be influenced not only by the concentration of endogenous ligand but the isoform as well.

Table of Links

Targets
GPCRs
LPA receptors http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=36
LPA$_1$ receptor http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=272
LPA$_2$ receptor http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=273
LPA$_3$ receptor http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=274
LPA$_4$ receptor http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=94
LPA$_5$ receptor http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=124
LPA$_6$ receptor http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=163

Ligands
LPA http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2906
These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al*., 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al*., 2015).

Introduction

Lysophosphatidic acid (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate; LPA) belongs to a family of endogenous lipid molecules involved in a number of cellular processes including proliferation, apoptosis and migration. LPA exerts these biological effects through highly efficacious interactions with its corresponding G protein-coupled receptors (GPCRs), the LPA family of receptors, leading to downstream intracellular signalling events. The most widely expressed is the LPA$_1$ receptor, which predominantly couples to G$_{q/11}$ and G$_{i/o}$, as well as G$_{12/13}$, leading to calcium mobilization and the inhibition of cAMP generation (Alexander *et al.,* 2015). This can lead to diverse cellular processes such as cell proliferation, actin cytoskeletal rearrangement and neurite retraction (Contos *et al.,* 2000).

Several molecular species or analogues of LPA exist which have varying carbon chain lengths and degrees of saturation, and these LPA analogues have been detected in biological fluids such as serum and plasma, linked to either the *sn-1* or *sn-2* glycerol backbone. The major analogues of LPA found in human blood are the 16-, 18- and 20- long acyl chain lengths, with 86 % of LPA in serum consisting of 18:1, 18:2 and 20:4 LPA. These are found at high nanomolar concentrations in the plasma but can exceed 10 µM in serum (Baker *et al.,* 2001, Lin *et al.,* 2010).

The LPA$_1$ receptor predominantly signals through G$_{i/o}$ and G$_{q/11}$ proteins to increase levels of intracellular calcium, but has also been shown to couple to G$_{12/13}$ (Fukushima *et al.,* 1998, Alexander *et al.,* 2015). This promiscuous coupling means that agonists at the LPA$_1$ receptor have the potential to demonstrate signalling bias, if they stabilise distinct conformations of the receptor that can differentially activate distinct G proteins leading to disparate functional end points. This phenomenon of agonist-biased signalling has been described for a number of different synthetic ligands at GPCRs, where the endogenous ligand is used as the un-biased comparator ligand (for reviews see: Rajagopal *et al.,* 2010, Kenakin and Miller, 2010). To date the majority of these studies have been performed in cell-lines that recombinantly express receptors of interest (Berg *et al.,* 1998, Gay *et al.,* 2004, Cordeaux *et al.,* 2001, Lane *et al.,* 2007, Rosethorne and Charlton, 2011). These are useful systems to examine signal bias as responses can be directly attributed to the receptor being over-expressed; however, some receptors have also been shown to elicit different responses dependent upon the cell type they are expressed in due to the presence of a different complement of signalling molecules. For example studies in mammalian cells indicate that unsaturated LPA analogues have lower EC$_{50}$ values than fully saturated analogues (Fujiwara *et al.,* 2005), whereas in human A431 cells no

correlation between degree of saturation and potency was observed (Jalink *et al.,* 1995). The different analogues of LPA will possess a range of physicochemical properties, depending upon the structure of the acyl chain, most notably in their solubility and tendency to form micelles. This may contribute to the range of biological activities that have been observed for these molecules. The vast diversity of the LPA analogues and the range of signalling molecules available to the LPA$_1$ receptor therefore represents a useful system in which to examine agonist-bias from endogenous ligands.

We therefore aimed to determine whether any signal bias exists with the different LPA analogues acting at an endogenously expressed LPA$_1$ receptor in primary human lung fibroblasts (HLF). This may have implications in patho-physiological processes as the activation of different G proteins by the range of endogenous ligands present in serum and plasma, may result in diverse cellular responses.

MATERIALS & METHODS

LPA analogues

LPA analogues tested were purchased from Avanti Polar Lipids (Alabaster, USA) or Echelon Biosciences (Utah, USA). Purity of the LPA analogues was stated to be >99 % LPA (see Supplementary table 1 for details).

All stock solutions were made up to 10 mM in water and stored at -20 °C.

For agonist treatments, all LPA analogues were prepared in Hanks' balanced salt solution (HBSS) with Ca^{2+}/Mg^{2+}, containing 20 mM HEPES, 0.1 % (w/v) BSA.

Cell culture and maintenance of HLF

HLF were maintained in DMEM containing high glucose and HEPES, supplemented with heat inactivated foetal bovine serum (FBS; 10 % v/v), penicillin (100 $iu.mL^{-1}$), streptomycin (100 $\mu g.mL^{-1}$), and sodium pyruvate (1 mM). Starve medium was the same as growth medium but devoid of FBS.

Calcium Mobilization assay

Normal primary HLF were plated in 384-well black walled, clear bottom plates at a density of 8,000 $cells.well^{-1}$. Cells were incubated for 24 h at 37°C/5 % CO_2 in a humidified atmosphere. After this time, spent media was removed, replaced with serum free media and cells incubated for a further 24 h. To monitor changes in intracellular calcium, the FLIPR® Calcium 4 Assay Kit (Molecular Devices, Sunnyvale, USA) was used. The dye was made up in HBSS with Ca^{2+}/Mg^{2+}, containing 20 mM HEPES, 0.1 % (w/v) BSA, 2.5 mM probenecid, 0.2 % (w/v) pluronic acid. On the day of experiment, calcium 4 dye, was added to the cell plate and incubated for 45 min at 37°C/5 % CO_2. For inhibition assays, antagonist was pre-incubated with cells for 30 min at 37°C/5 % CO_2. Agonist was added online using the Functional Drug Screening System 7000 (FDSS7000; Hamamatsu Photonics, Hertfordshire, UK) and fluorescence monitored for 5 min with 1s increments. Peak responses were taken for data analysis (between 2-3 minutes).

For $G_{i/o}$ versus $G_{q/11}$ experiments, cells were pre-treated with 200 ng/mL pertussis toxin (PTx) during the starvation step to inhibit $G_{i/o}$-dependent signalling. Calcium mobilisation was then carried out as described above.

ERK Imaging Assay

Normal primary HLF were plated at 3,000 cells.well^{-1} in 384-well black walled, clear bottom plates. Cells were incubated for 24 h at 37°C/5 % CO_2, after which time spent media was removed, replaced with serum free media and cells incubated for a further 24 h. Cells were then incubated with agonists for 5 min at 37°C/5 % CO_2. For IC_{50} determinations, antagonist was added to the cells 30 min prior to agonist addition. After stimulation, cells were fixed in 4 % paraformaldehyde for 20 min, washed 2x in PBS (with Ca^{2+}/Mg^{2+}), and incubated with permeabilising blocking buffer (dPBS (with Ca^{2+}/Mg^{2+}), 10 % FBS (v/v), 0.1 % Tween-20 (v/v)) for 1 h at 37°C. Wells were then washed 3x in wash buffer (TBS-T: Tris Buffered Saline (TBS), 0.05% (v/v) Tween-20) and incubated with the phospho-p44/42 ERK1/2 (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb primary antibody (1:1000 dilution) at 4°C overnight. Following 3x wash in TBS-T, wells were incubated with blocking buffer containing Hoechst (2 µM), and CF™647-conjugated Affini-pure goat anti-rabbit IgG (1:1000 dilution) for 1 h at 37°C. Wells were washed 3x in TBS-T to remove secondary antibody and cell plate imaged using the InCell 2000 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) using DAPI filter (0.1 sec exp) for nuclei and Cy5 filter (1.3 sec exp) to monitor ERK1/2 phosphorylation (pERK). Images were analysed for cell intensity using the INCell Analyser software. To quantify levels of pERK fluorescence, a standard Multi-Target Analysis (MTA) algorithm was used in the INCell Analyzer Workstation (v3.7.1). Nuclei were defined using Top-hat segmentation, with a minimum area of 80 µm^2 (sensitivity 50). Cells were defined using Multiscale Top-hat segmentation with a characteristic area of 300 µm^2 (sensitivity 58).

For $G_{i/o}$ versus $G_{q/11}$ experiments, cells were pre-treated with 200 ng/mL PTx during the starvation step, to inhibit $G_{i/o}$-dependent signalling. ERK phosphorylation assay was then performed as described above.

Data Analysis

Graphs were fitted to data and statistical analysis performed using GraphPad Prism (V7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Results are expressed as the mean ± standard error of mean (SEM) from three separate experiments, run in duplicate. Concentration-response data were fitted using a four-parameter logistic equation. IC_{50} values obtained from the inhibition curves were converted to K_i values using the method of Cheng and Prusoff (1973). The Pearson correlation coefficient (r) was determined using the standard correlation function in GraphPad Prism, followed by a two-tailed t test to determine significance. All statistical tests used $P \leq 0.05$ as critical level of significance.

The ability of an agonist to cause a functional response after binding to a receptor is known as the transduction coefficient (transduction ratio) and is a function of both the binding affinity (K_A) and the efficacy (τ) of the ligand. To compare agonist responses between different assays, the $Log(\tau/K_A)$ or LogR was calculated using the Operational model of agonism (for details see van der Westhuizen *et al.* (2014)). For the purposes of this analysis, only the log of the ratio (LogR) between K_A and τ ($Log(\tau/K_A)$) was calculated for each agonist at each pathway, as bias can arise through differences in either affinity or efficacy. The responses to all agonists at each pathway were globally fitted with the parameters of basal, E_{max} and n shared for all agonists. For full agonists, K_A was constrained to > 0, and estimated directly from curve fitting for partial agonists.

For estimation of observational bias for the different assays, the LogR for each compound was compared to the LogR for the 'neutral' compound 18:1 LPA (or 17:0), using equation (1).

$$\Delta LogR = \Delta log \left(\frac{\tau}{K_A}\right) = log \left(\frac{\tau}{K_A}\right)_{Ligand} - log \left(\frac{\tau}{K_A}\right)_{Reference} \tag{1}$$

Ligand bias ($\Delta\Delta LogR$) was calculated using the difference between the $\Delta LogR$ values derived for each pathway using equation (2), and from this the bias factor for each compound was calculated from equation (3).

$$\Delta\Delta LogR = \Delta LogR_{L1:P1} - \Delta LogR_{L1:P2} \tag{2}$$

where L1 is ligand 1, P1 is pathway 1, L2 is ligand 2, and P2 is pathway 2.

$$\text{Bias factor} = 10^{\Delta\Delta LogR} \qquad OR \qquad 10^{\Delta\Delta Log\left(\frac{\tau}{K_A}\right)} \tag{3}$$

Reagents

Primary HLF were purchased from PromoCell (Heidelberg, Germany) and Lonza (Slough, UK). CF™647-conjugated Affini-pure goat anti-rabbit IgG was purchased from Biotium (Cambridge, UK) and phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb primary antibody was purchased from Cell Signalling technology (Hitchin, UK). 384-well GPCR TaqMan arrays, RNAqueous kit, TURBO DNase, High Capacity RNA-to-cDNA kit, Hoechst, cell media and growth supplements were all purchased from Invitrogen Life Technologies (Paisley, UK). FLIPR® Calcium 4 Assay Kit was purchased from Molecular Devices (Sunnyvale, USA). AM966 was synthesized in-house.

RESULTS

LPA receptor expression

To determine which of the LPA receptors are present at significant levels in HLF, a Taqman assay quantifying the endogenous expression of the different LPA receptors was performed. The relative expression of each receptor was compared to the house keeping gene HPRT1. The LPA_1 receptor had the highest level of expression in the HLF (4.4 ±1.5 fold relative expression compared to HPRT; Supplementary figure 1), while the other receptors tested had no measurable expression in these cells (< 0.1 fold relative expression compared to HPRT). Based on the expression data we used the HLF to investigate LPA_1 receptor mediated signalling of different molecular analogues of LPA.

Calcium mobilization

To investigate the intracellular signalling downstream of the LPA_1 receptor, we first monitored calcium mobilization in HLF after treatment with a range of concentrations of eleven LPA analogues (see Supplementary table 1 for chemical structures). These eleven analogues can be divided into four sub groups of short chain (14:0, 16:0 and 17:0), 18:x, 20:x and ester C18:x LPA. The most quantitatively abundant forms of LPA in human plasma are 16:0, 18:2, and 18:1 LPA. The latter form is perhaps the most commonly used laboratory reagent for signalling studies, therefore it was used as a positive control in these experiments (Choi *et al.,* 2010).

All LPA analogues were able to increase intracellular calcium mobilization in a concentration dependent manner, with a range of potencies and intrinsic activities (figure 1). Of these, C18:1 was the most potent form of LPA, despite having modest intrinsic activity (77 %), with the fully saturated 20:0, C18 and 17:0 the least potent. All analogues except for 20:4, 18:3, C18:1, C18 and 20:0 were fully efficacious, with a comparable E_{max} to 18:1 LPA (table 1). Among the 18: LPAs tested a general trend was observed where an increase in the degree of saturation of the 18: LPAs resulted in an increase in both potency and intrinsic activity in the calcium mobilization assay. In contrast, among the 20:x and C18:x LPAs tested, the fully saturated 20:0 and C18:0 were the least potent and gave only partial responses in this assay.

ERK phosphorylation

As both $G_{q/11}$ and $G_{i/o}$ have been shown to activate ERK (Goldsmith and Dhanasekaran, 2007), we explored the ability of the same range of LPA analogues to phosphorylate ERK in the primary HLF. In order to determine the correct incubation time for this assay, we first

performed a time course of ERK phosphorylation with 18:1 LPA (data not shown). The pERK response was rapid and short-lived, with maximal response achieved between 2 – 5 minutes after which time signal returned to basal levels. For this reason, all further experiments were performed after 5 minute stimulation.

All of the LPA analogues were able to promote ERK phosphorylation with a range of intrinsic activities and potencies (figure 2). 18:1 LPA gave the largest maximal response, so this was taken to be 100 % in these assays. In comparison, all other analogues of LPA tested were partial agonists in the pERK assay, with maximal responses ranging from 25 – 80 % of that of 18:1 LPA (table 1). 20:0 and C18 were the least active LPA analogues in the pERK assay, able to only partially phosphorylate ERK at the highest agonist concentrations. Generally most analogues of LPA had either similar (18:1, 18:3, 20:4) or slightly lower (5 – 10-fold) potency in the pERK compared to the calcium mobilization assay, however 17:0 was more potent at pERK than calcium and C18:1 was more than 20-fold more potent in the calcium assay than pERK. This suggests there may be some bias in the ability of different analogues of LPA to signal to the two different pathways.

Quantification of ligand bias

Without truly understanding the difference in system and observational bias between the two assays, it is difficult to use agonist potency to determine which of the LPA analogues are biased and which are non-biased for the different systems being studied. For this reason, we attempted to quantify the level of ligand bias between the two pathways (calcium mobilization versus ERK phosphorylation) using the operational model to calculate LogR or Log(τ/KA) for each ligand (van der Westhuizen *et al.,* 2014, Kenakin and Christopoulos, 2013). We then calculated the ΔLogR for each ligand, using 18:1 as the control ligand as this is one of the most abundant LPA analogues in the body, and was the only agonist that gave a maximal response in both assays (figure 3; Table 1). Comparison of the ΔLogR of each analogue of LPA between the two different assay formats revealed significant differences in the ΔLogR values between assays for 16:0, 17:0, 18:2 and C18:1 LPA ($p < 0.05$, unpaired t-test), which were selected for further investigation, using comparison 18:1 LPA as the non-biased control ligand.

In order to confirm that the responses observed were solely due to activation of the LPA$_1$ receptor, we tested this subset of LPA analogues against the selective LPA$_1$ receptor antagonist (4′-{4-[(R)-1-(2-chloro-phenyl)-ethoxycarbonylamino]-3-methyl-isoxazol-5-yl]-biphenyl-4-yl)-acetic acid (AM966) (Choi *et al.,* 2010, Swaney *et al.,* 2010). AM966 was able to

completely inhibit both calcium and pERK responses elicited by 18:1, 16:0, 17:0, 18:2 and C18:1 LPA (figure 4), demonstrating that the calcium response observed is solely due to activation of the LPA$_1$ receptor. IC$_{50}$ values derived from this assay were converted into K_i using the Cheng-Prusoff equation (table 2).

Effect of Pertussis toxin on calcium and pERK responses

It has been demonstrated previously that the LPA$_1$ receptor can couple to multiple pathways (Alexander *et al.,* 2015), of which both G$_{i/o}$ and G$_{q/11}$ are capable of causing increases in intracellular calcium release and ERK phosphorylation. We hypothesized that the bias we observed between calcium mobilization and ERK phosphorylation for 16:0, 17:0, 18:2 and C18:1 LPA may be due to activation of different G proteins. To explore this further, the degree of calcium mobilization and ERK phosphorylation was determined after pre-treatment of cells with PTx to inhibit G$_{i/o}$ proteins.

All LPA analogues tested were less potent in the calcium mobilization assay after 24 h treatment with PTx, and demonstrated reduced intrinsic activity. In contrast, we were unable to accurately determine EC$_{50}$'s in the ERK phosphorylation assay after PTx treatment, as pERK responses were almost completely abolished for all the analogues of LPA tested (figure 5; table 3). From these data we can infer that the calcium response is dependent on the activation of multiple G proteins, whereas the pERK response is predominantly PTx-sensitive. To explore the bias we observed for 16:0, 17:0, 18:2 and C18:1 LPA further, we wanted to quantify the signal bias between the total pERK response and the PTx-insensitive calcium response (data from experiments performed in the presence of PTx).

To do this we fitted these data to the Operational model to calculate ΔΔLogR for each ligand between the two different pathways (for details see van der Westhuizen *et al.* (2014)). The results from this analysis (figure 6a; table 4) demonstrate that for some of the molecular analogues a bias for one particular signalling pathway does exist. Statistical analysis of the bias factors between both pathways, when compared to 18:1 LPA, showed that a significant difference was seen for 18:2 and 17:0 LPA, both of which appeared more biased towards the pERK pathway.

DISCUSSION

The LPA$_1$ receptor is widely expressed in the body, and has been implicated in diverse cellular and physiological responses, such as proliferation and survival, neurite retraction, brain development, renal and pulmonary fibrosis (Fukushima *et al.,* 1998, Estivill-Torrus *et al.,* 2008, Tager *et al.,* 2008, Pradere *et al.,* 2007). The endogenous ligand, LPA, has many different naturally occurring analogues which differ in chain length or saturation, and are found within a range of biological fluids including serum and blood. The physiological relevance of this diversity is not fully appreciated, however it may play an important role in pathology with evidence demonstrating that certain analogues may be differentially regulated in disease. For example, levels of the polyunsaturated 22:5 and 22:6 LPA are increased after allergen challenge in asthmatics (Park *et al.,* 2013), and IPF patients demonstrated significantly higher levels of 22:4 LPA in their exhaled breath condensate compared to controls (Montesi *et al.,* 2014). It has also been demonstrated that there is a preference for polyunsaturated LPAs in the follicular fluid of patients undergoing *in vitro* fertilization (Yamamoto *et al.,* 2016). In addition, 18:0, 18:1 and 16:0 LPA have been shown to increase in mouse spinal dorsal horn after nerve injury, with 18:1 LPA being the key species associated with amplification of LPA production in peripheral neuropathic pain (Ma *et al.,* 2013). The way in which these different analogues are regulated and signal within the body after activation of the LPA$_1$ receptor is still relatively poorly understood.

As well as having a number of endogenous LPA analogues to regulate LPA$_1$ receptor signalling, the receptor itself can also couple to multiple signalling pathways, including G$_{i/o}$, G$_{q/11}$ and G$_{12/13}$ proteins, to initiate diverse cellular responses (Fukushima *et al.,* 1998). For example, LPA$_1$ receptor activation leads to ERK phosphorylation via the G$_{i/o}$-Ras leading to gene transcription, cell cycle progression and proliferation (Kranenburg and Moolenaar, 2001). Fibroblast chemotaxis to LPA has also been shown to occur via the PTx-sensitive G$_{i/o}$ proteins (Tager *et al.,* 2008), whereas G$_{12/13}$-RhoA pathway is responsible for actin cytoskeleton rearrangements in fibroblasts (Sakai *et al.,* 2013). In addition, it has been demonstrated that the LPA receptor can transactivate the epidermal growth factor (EGF) receptor, leading to ERK phosphorylation via G$_{i/o}$-derived βγ sub units (Daub *et al.,* 1996, Tveteraas *et al.,* 2016). In this study we have investigated a number of different naturally occurring analogues of LPA, after activation of an endogenous LPA$_1$ receptor in primary HLF. In addition to calcium mobilization, we have explored the ability of these ligands to activate the Ras-Raf-ERK signalling cascade resulting in ERK phosphorylation, which may occur downstream of either

$G_{i/o}$ or $G_{q/11}$ proteins (Goldsmith and Dhanasekaran, 2007), or via G protein-independent mechanisms involving β-arrestin (Lefkowitz and Shenoy, 2005, Rosethorne and Charlton, 2011). The signalling properties of the different LPA analogues were first characterized in the two assays, and PTx treatment used to determine the relative contributions of $G_{i/o}$ and $G_{q/11}$ ($G_{12/13}$) proteins.

All analogues of LPA were capable of activating both pathways, with 18:1 LPA being the only fully efficacious agonist in both assays. The key difference observed in the responses between the assays was that all LPA analogues except 17:0 LPA were less potent in the pERK assay than the calcium mobilization assay.

In order to understand these differences, we first determined that all responses were due to only the LPA_1 receptor. Using the selective LPA_1 receptor antagonist AM966, we demonstrated that all responses were solely due to the activation of the LPA_1 receptor. We also observed that there were significant differences between the K_i values determined from the two assays, with AM966 demonstrating consistently higher affinity at the pERK pathway when compared to calcium (2-7 fold). This is most likely due to complications arising from non-equilibrium conditions in the rapid calcium signalling system. As the calcium assay is measured earlier than the pERK, it is likely that we are slightly underestimating the affinity of AM966 at this pathway due to these hemi-equilibrium conditions (Charlton and Vauquelin, 2010).

All responses were AM966-dependent, suggesting that bias exists between the two signalling pathways downstream of the LPA_1 receptor. In order to explore the potential bias we have attempted to control for differences in the kinetics of the different assays by taking the initial peak response for both assays. This occurred between 2-3 minutes for the calcium response and at 5 minutes for the pERK response. Recent data have demonstrated that LPA_1 receptor phosphorylation occurs after 15-30 minute treatment with LPA, with internalization occurring after 30-60 minutes (Alcántara-Hernández, 2015). Although we cannot completely rule out a contribution of these processes, this suggests that the responses we are measuring will not be affected by differences in receptor state, but are in fact due to differential activation of the pathways being measured.

In order to quantify the potential bias demonstrated by the LPA analogues, functional assays for each pathway must be used, and the differences between kinetics, receptor reserve, signal amplification etc. taken into account. The most common way to do this is to use a reference (usually endogenous) agonist that is assumed to be non-biased, and relate experimental data to

that obtained for the reference ligand. As all the ligands we have tested here are endogenous LPAs for the LPA_1 receptor, we have chosen to use 18:1 LPA as the control agonist, as it was the only analogue that was fully efficacious in both assays. We used the Operational model of agonism to calculate a LogR for each ligand, which is a combination of efficacy (τ) and affinity (K), both of which can contribute to ligand bias in a functional system. By comparing the LogR of each analogue to our control analogue, 18:1, we can eliminate any bias that may exist due to differences in the efficiency of receptor coupling to each second messenger. From this analysis, we chose to further explore the signalling profiles of 16:0, 17:0, 18:2 and C18:1 LPA, as they yielded LogR values that were significantly different between the two assay formats, indicating that they may be biased agonists.

For these selected agonists, we wanted to further explore the signal bias, by determining the role of $G_{i/o}$ proteins in each of the responses we have measured. To do this we used PTx treatment to inactivate the $G_{i/o}$ subset of G proteins to determine what effect this would have on the calcium and pERK responses. ERK phosphorylation was almost completely abolished after PTx treatment, while a significant proportion of the calcium response remained. This suggests that the calcium response is due to the activation of multiple G proteins, whereas the pERK response is predominantly PTx-sensitive and therefore may be due to $G_{i/o}$ protein activation.

For this reason, we repeated the LogR analysis using data for ERK phosphorylation (without PTx treatment) compared to the PTx-treated calcium responses. From these data we observed a significant difference between the $\Delta\Delta$LogR for 18:2 and 17:0 LPA, compared to 18:1, our control LPA, suggesting that these ligands are more biased towards the pERK pathway over the PTx-insensitive calcium response when compared to 18:1 LPA. To confirm that the ERK phosphorylation is occurring independent of $G_{q/11}$ activation, and that the PTx-treated calcium responses are solely due to $G_{q/11}$ activation, it would be interesting to repeat these experiments in the presence of selective G_q inhibition, or down-regulation of specific G proteins using siRNA treatment. Although we cannot rule out a role for LPA_1 receptor mediated EGF transactivation leading to ERK phosphorylation, this has been shown to be dependent upon $G_{i/o}$-derived βγ subunits in fibroblasts, and therefore this would still indicate ligand bias at the level of the G protein (Daub *et al.,* 1996, Tveteraas *et al.,* 2016).

In summary, we have characterized the calcium and ERK responses downstream of the LPA_1 receptor for a number of different LPA molecular analogues, and demonstrated that there is signalling bias between these pathways in HLF. As these experiments were performed in a

native cell background almost exclusively expressing the LPA$_1$ receptor, using a range of endogenous ligands, they could be indicative of the native *in vivo*/clinical situation in the lung. The importance of LPA as a key signalling molecule is apparent due to its widespread occurrence in biological fluids and in majority of the tissues in the body. It is also becoming increasingly important for drug discovery efforts and has been shown to play an important role in the pathogenesis of a range of diseases, with fibrosis, rheumatoid arthritis, neuropathic pain, cardiovascular disease and cancer being key areas of interest (Choi *et al.,* 2010, Tigyi, 2010, Yanagida and Ishii, 2011, Lin *et al.,* 2010, Velasco *et al.,* 2016). Due to the diversity in the LPA analogues, it is possible that levels of specific molecular analogues of LPA are deregulated in certain conditions and the ligand bias could be explored in developing selective ligands for therapeutic applications. Here we have investigated signalling bias for the LPA$_1$ receptor between calcium mobilization and ERK phosphorylation, however there is also the potential for signalling through G protein-independent pathways such as β-arrestin. These data were all generated in human primary cells which endogenously express the LPA$_1$ receptor, as well as the relevant effector proteins. For this reason we believe the bias we have detected may be more relevant for therapeutic applications. Unfortunately it is still difficult to monitor β-arrestin recruitment or receptor internalization without the need to transfect labelled proteins which may predispose the signalling towards these over-expressed pathways. For this reason we have not monitored these pathways in this study. Together these data demonstrate the importance in considering not only the LPA isoform associated with health and disease, but the receptor signalling pathways activated as well.

AUTHOR CONTRIBUTIONS

Participated in research design: Sattikar, Rosethorne, Dowling

Conducted experiments: Sattikar

Contributed new reagents or analytic tools:

Performed data analysis: Sattikar, Rosethorne

Wrote or contributed to the writing of the manuscript: Sattikar, Rosethorne, Dowling

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Table 1   Potency and intrinsic activity for different molecular species of LPA in different functional assays.

	Calcium mobilization			ERK phosphorylation		
Ligand	**pEC$_{50}$**	**Intrinsic activity**	**ΔLogR**	**pEC$_{50}$**	**Intrinsic activity**	**ΔLogR**
18:1 LPA	7.73 ± 0.12	101.4 ± 5.83	0	7.51 ± 0.08	105.3 ± 4.31	0
14:0 LPA	7.26 ± 0.11	101.4 ± 3.74	-0.62 ± 0.10	6.72 ± 0.04	73.55 ± 4.85	-0.78 ± 0.07
16:0 LPA*	7.32 ± 0.16	104.7 ± 4.71	-0.18 ± 0.10	6.58 ± 0.10	76.45 ± 6.40	-1.13 ± 0.09
17:0 LPA*	6.44 ± 0.05	94.48 ± 10.6	-1.39 ±0.10	7.14 ± 0.06	79.81 ± 5.22	-0.20 ±0.09
18:2 LPA*	6.84 ± 0.03	112.9 ± 9.93	-0.90 ± 0.10	6.49 ± 0.04	79.45 ± 2.88	-1.36 ± 0.09
18:3 LPA	6.45 ± 0.06	82.01 ± 2.27	-1.52 ± 0.12	6.31 ± 0.10	78.85 ± 3.41	-1.29 ± 0.17
20:0 LPA	5.44 ± 0.24	58.83 ± 2.16	-2.65 ± 0.16	ND	32.89 ± 1.93	-2.56 ± 0.10
20:4 LPA	6.85 ± 0.22	89.36 ± 6.72	-1.09 ± 0.12	6.63 ± 0.24	50.53 ± 2.52	-1.09 ± 0.13
C18 LPA	6.22 ± 0.05	66.67 ± 5.98	-1.98 ± 0.14	ND	26.67 ± 1.13	-2.34 ± 0.31
C18:1 LPA*	8.04 ± 0.18	77.04 ± 6.36	0.04 ± 0.12	6.70 ± 0.12	78.06 ± 5.49	-0.99 ± 0.09

Potency and intrinsic activity values for LPA$_1$ receptor agonists in calcium mobilization and ERK phosphorylation assays in HLF. Intrinsic activity was calculated as a percentage of the maximal 18:1 LPA response. ΔLogR were calculated for each agonist using the Operational model of agonism (van der Westhuizen, 2014). Data were analysed in a pairwise manner using a two-tailed unpaired Student's t test to determine if there was a significant difference in the ΔLogR values between the two assays. Data are expressed as means ± SEM for 3 independent experiments.

*ND – not determined due to incomplete curve   * p < 0.05*

Table 2   Affinity values for the LPA$_1$ antagonist AM966 versus different LPA$_1$ agonists.

	Calcium mobilization (pK_i)	ERK phosphorylation (pK_i)
18:1 LPA*	7.85 ± 0.03	8.37 ± 0.07
16:0 LPA*	7.73 ± 0.05	8.13 ± 0.13
17:0 LPA*	7.77 ± 0.02	8.62 ± 0.10
18:2 LPA*	7.86 ± 0.02	8.68 ± 0.02
C18:1 LPA*	7.78 ± 0.02	8.33 ± 0.04

Affinity of LPA$_1$ receptor antagonist AM966 in calcium mobilization and ERK phosphorylation assays after treatment with a range of different LPA isoforms. Data were analysed in a pairwise manner using a two-tailed unpaired Student's t test to determine if there was a significant difference in the affinity values between the two assays. Data are shown as means ± SEM for three independent experiments.

*$P < 0.05$

Table 3   Effect of PTx treatment on the potency and intrinsic activity of different LPA$_1$ receptor agonists.

	Calcium mobilization				ERK phosphorylation			
	Control		PTx treated		Control		PTx treated	
Ligand	pEC$_{50}$	Intrinsic activity	pEC$_{50}$	Intrinsic activity	pEC$_{50}$	Intrinsic activity	pEC$_{50}$	Intrinsic activity
16:0 LPA	7.28 ± 0.07	96.7 ± 5.06	6.40 ± 0.09	58.6 ± 1.99	6.80 ± 0.09	77.9 ± 6.51	ND	13.3 ± 3.17
17:0 LPA	6.84 ± 0.03	97.0 ± 4.76	6.58 ± 0.06	68.0 ± 4.32	7.16 ± 0.04	79.4 ± 0.82	ND	15.0 ± 6.54
18:2 LPA	6.84 ± 0.08	96.1 ± 3.15	6.37 ± 0.03	70.2 ± 0.48	6.65 ± 0.05	80.2 ± 2.72	ND	20.9 ± 3.00
C18:1 LPA	7.27 ± 0.02	75.4 ± 1.11	6.74 ± 0.04	48.7 ± 1.03	6.58 ± 0.15	78.4 ± 0.17	ND	16.5 ± 4.01
18:1 LPA	7.77 ± 0.07	100	6.84 ± 0.09	60.3 ± 2.42	7.43 ± 0.17	100	ND	5.69 ± 1.35

Potency and intrinsic activity of LPA$_1$ receptor agonists in calcium mobilization and ERK phosphorylation assays in HLF, with or without PTx treatment. Intrinsic activity was calculated as a percentage of the maximal 18:1 LPA response. Data are shown as means ± SEM for three independent experiments.

ND – not determined due to incomplete curve

Table 4 ΔΔLogR ratios and bias factors (BF) for selected LPA molecular species at the LPA_1 receptor.

	ΔlogR (18:1) calcium	ΔlogR (18:1) pERK	ΔΔLogR (18:1) (BF)
18:1 LPA	0	0	0 (1)
17:0 LPA	0.08 ± 0.11	-0.78 ± 0.19	0.87 ± 0.18 (7.35)*
18:2 LPA	-0.14 ± 0.11	-1.01 ± 0.21	0.86 ± 0.22 (7.31)*
16:0 LPA	-0.25 ± 0.11	-0.88 ± 0.21	0.63 ±0.24 (4.30)
C18:1 LPA	-0.27 ± 0.14	-0.47 ±0.19	0.20 ± 0.24 (1.59)

Transduction ratios (LogR) and Bias factors (BF) were calculated using the equations described in (van der Westhuizen *et al.*, 2014) from data plotted using the Operational Model. Data used were obtained from calcium experiment performed in the presence of PTx ($G_{q/11}$-dependent) and ERK phosphorylation performed in the absence of PTx ($G_{i/o}$-dependent), and calculations performed using either 18:1 or 17:0 LPA as the reference ligand. Data are expressed as the mean ± SEM of 3 independent experiments. Data were analysed in a pairwise manner using a two-tailed unpaired Student's t test [on ΔΔLogR] to determine the significance of the ligand biases.

*BF= Bias factor   *P <0 .05*

Figure Legends

Figure 1 Concentration-response curves for calcium mobilization in HLF using a) short chain b) 18:x, c) 20:x and d) C18:x molecular analogues of LPA. Data were normalised to the 18:1 LPA response and are expressed as means ± S.E.M. for three independent experiments.

Figure 2 Concentration-response curves for ERK phosphorylation in HLF using a) short chain b) 18:x, c) 20:x and d) C18:x molecular analogues of LPA. Cell intensity was normalised to the 18:1 LPA response and data expressed as means ± S.E.M. for three independent experiments.

Figure 3 Correlation between ΔLogR for calcium versus ERK phosphorylation in HLF. A Pearson correlation coefficient (r) was determined followed by a two-tailed t test to determine significance ($P \leq 0.05$).

Figure 4 Concentration-response curve to the selective LPA_1 receptor antagonist AM966, in the presence of an EC_{80} concentration of 16:0, 17:0, 18:2 & C18:1 LPA for a) calcium mobilization or b) ERK phosphorylation. Data are normalised to the EC_{80} control for each of the different LPA analogues and are expressed as means ± S.E.M. for three independent experiments.

Figure 5 Effect of PTx on calcium mobilization and ERK phosphorylation by selected LPA analogues; a) 18:1 LPA, b) 16:0 LPA, c) 17:0 LPA, d) 18:2 LPA and e) C18:1 LPA. HLF were treated with 150 ng/ml PTx during the serum starvation and the response to selected LPA analogues was measured in calcium mobilization and ERK phosphorylation assay. Data are normalised to the 18:1 LPA response and are expressed as means ± S.E.M. for three independent experiments.

Figure 6 a) ΔΔLogR for each ligand calculated between the ERK response (-PTx) and the calcium response (+PTx), using either 18:1 LPA or 17:0 LPA as the control, non-biased ligand. b) Bias plot for calcium mobilization versus ERK phosphorylation. The response in the ERK phosphorylation assay (-PTx) was plotted as a function of the corresponding response in the calcium mobilization assay (+PTx), for selected analogues of LPA.
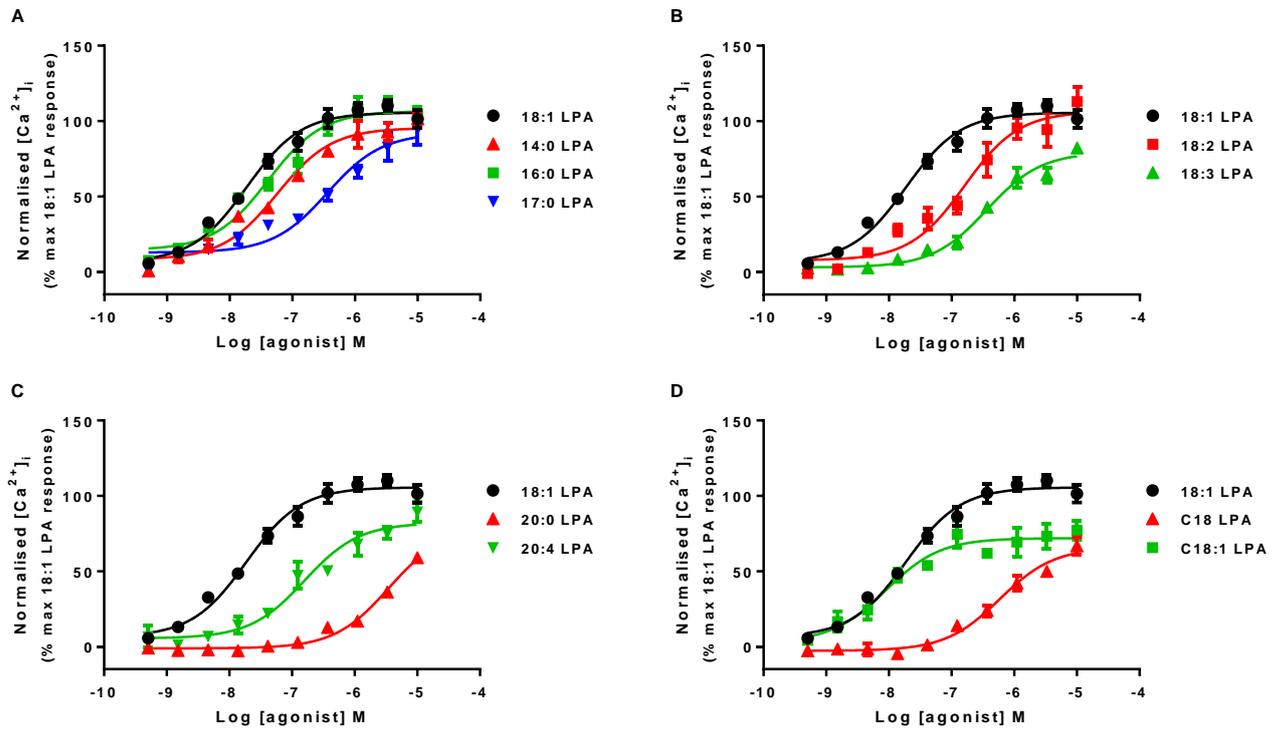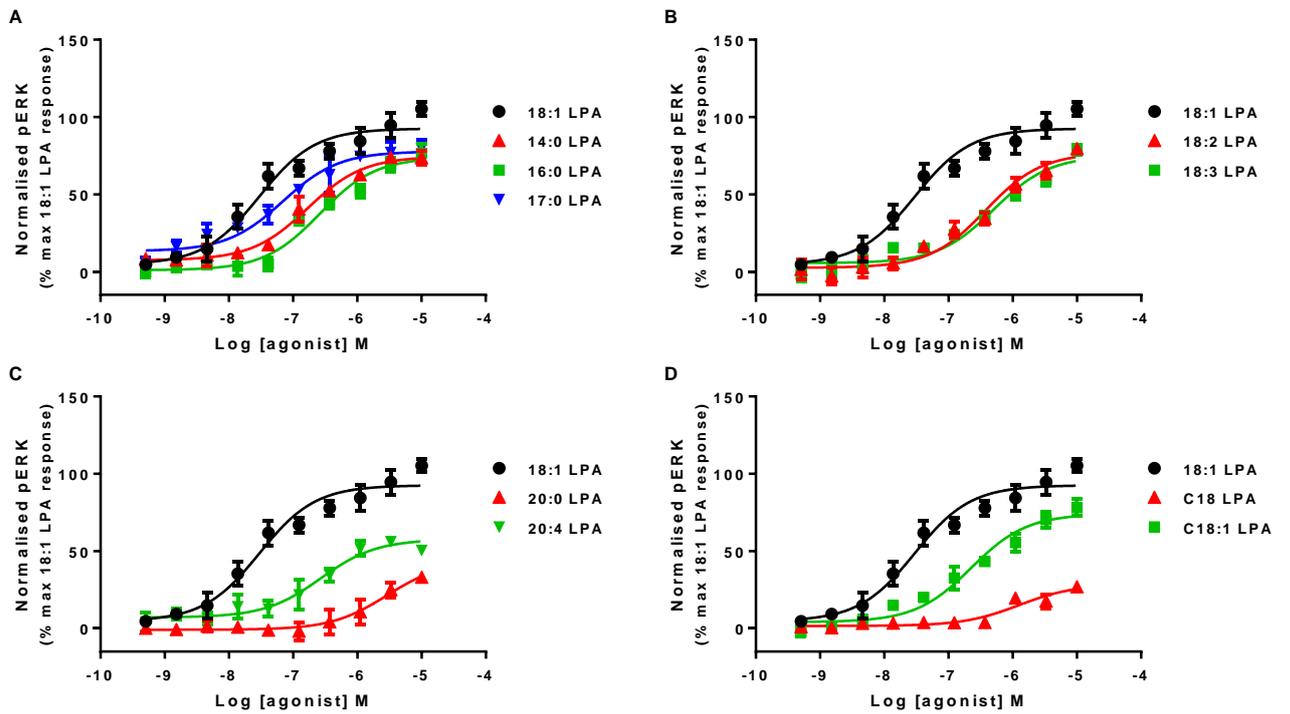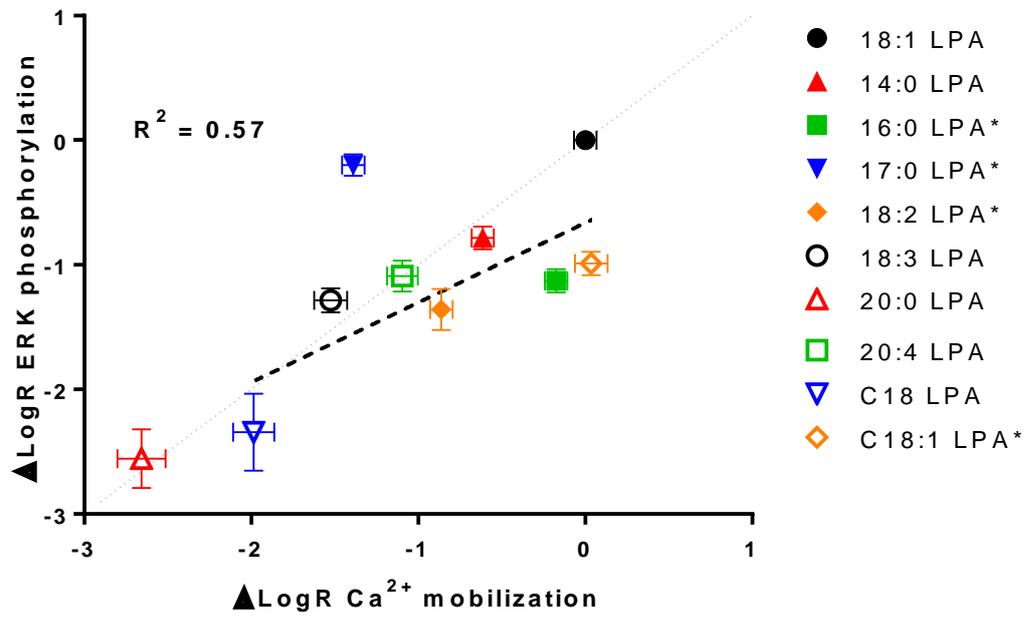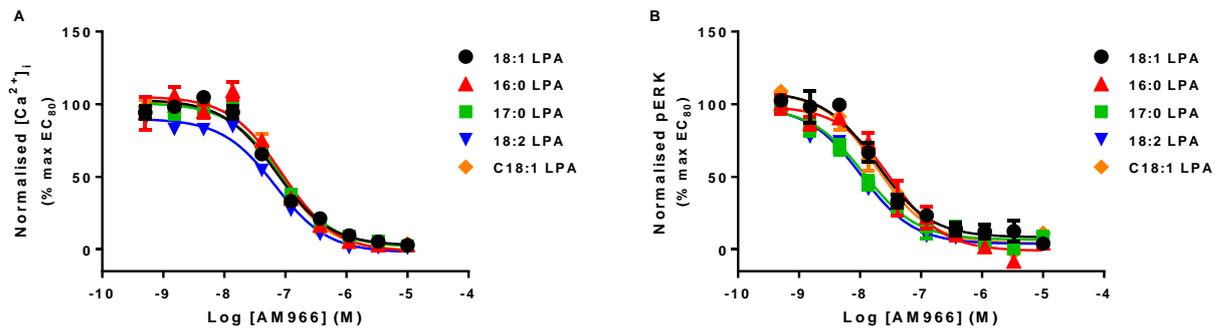
Figure 1

Figure 2

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