Lysophosphatidic acid-mediated Ca^{2+} mobilization in human SH-SY5Y neuroblastoma cells is independent of phosphoinositide signalling, but dependent on sphingosine kinase activation

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Extracellular application of lysophosphatidic acid (LPA) elevated intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in human SH-SY5Y neuroblastoma cells. The maximal response to LPA occurred between 0.1 and 1 μM , at which point $[\text{Ca}^{2+}]_i$ was increased by approx. 500 nM. This increase was of similar magnitude to that caused by the muscarinic acetylcholine receptor agonist methacholine (MCh), although the initial rate of release by LPA was slower. Both LPA and MCh released Ca^{2+} from intracellular stores, as assessed by inhibition of their effects by thapsigargin, a blocker of endoplasmic reticular Ca^{2+} uptake, and by the persistence of their action in nominally Ca^{2+} -free extracellular medium. Similarly, both agonists appeared to stimulate store-refilling Ca^{2+} entry. MCh produced a marked elevation in cellular $\text{Ins}(1,4,5)\text{P}_3$ and stimulated $[\text{^3H}]\text{InsP}$ accumulation in the presence of Li^+ . In contrast, LPA failed to stimulate detectable phosphoinositide turnover. Chronic down-regulation of $\text{Ins}(1,4,5)\text{P}_3$ receptor (InsP_3R) proteins with MCh did not affect Ca^{2+} responses to LPA. In addition, heparin, a competitive antagonist of InsP_3Rs , blocked Ca^{2+} -mobilization in permeabilized SH-SY5Y

cells in response to MCh or exogenously added $\text{Ins}(1,4,5)\text{P}_3$, but failed to inhibit Ca^{2+} -release induced by LPA. Elevation of $[\text{Ca}^{2+}]_i$ elicited by LPA was blocked by guanosine 5'-[β -thio]-diphosphate, indicating that this agonist acts via a G-protein-coupled receptor. However, pertussis toxin was without effect on LPA-evoked $[\text{Ca}^{2+}]_i$ responses, suggesting that $\text{G}_{i/o}$ -proteins were not involved. In the absence of extracellular Ca^{2+} , *N,N*-dimethylsphingosine (DMS, 30 μM), a competitive inhibitor of sphingosine kinase, blocked LPA-induced Ca^{2+} responses by almost 90%. In addition, MCh-induced Ca^{2+} responses were also diminished by the addition of DMS, although to a lesser extent than with LPA. We conclude that LPA mobilizes intracellular Ca^{2+} -stores in SH-SY5Y cells independently of the generation and action of $\text{Ins}(1,4,5)\text{P}_3$. Furthermore, the Ca^{2+} -response to LPA appears to be dependent on sphingosine kinase activation and the potential generation of the putative second messenger sphingosine 1-phosphate.

Key words: Ca^{2+} -signalling, dimethylsphingosine, heparin.

INTRODUCTION

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycerol-3-phosphate), in addition to playing a role in the *de novo* synthesis of phospholipids, demonstrates many properties of an extracellular signalling molecule [1–3]. LPA elicits a wide range of biological effects, including inhibition of adenylate cyclase, activation of phosphoinositide-specific phospholipase C (PLC), mobilization of intracellular Ca^{2+} stores, cell proliferation, neurite retraction and neurotransmitter release [4–10]. Radioligand binding studies [9,11] and molecular cloning [12–16] suggest that most of these actions of LPA are likely to be receptor-mediated.

Many studies have now demonstrated the ability of LPA to stimulate increases in intracellular free Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) [4,6,7,11,17–21], predominantly via the mobilization of Ca^{2+} stores. Similar to other agents acting through G-protein-coupled receptors (GPCRs) (see [22]), this action of LPA can occur through the production of the second messenger, $\text{Ins}(1,4,5)\text{P}_3$. Indeed, LPA stimulates InsP production in numerous cell types [4,6,11,18,20], and the Ca^{2+} response can be inhibited by the PLC inhibitor U73122 [6,19]. However, in many of the reports it is unclear which LPA receptor subtype is involved in the Ca^{2+} -

mobilizing response and also which G-protein is responsible for transducing the signal. Recently, Edg-2 and Edg-4 (putative GPCRs for LPA [14,15]) have been recombinantly expressed in cultured mammalian cells [21], the stimulation of which leads to InsP production and Ca^{2+} mobilization with both subtypes. The Edg-2-mediated response is abolished by pretreatment with pertussis toxin (PTX) or U73122, indicating that $\text{Ins}(1,4,5)\text{P}_3$ production is through PLC activation, most likely via $\beta\gamma$ -subunits of G_i -proteins. In contrast, the Edg-4 response is only partially inhibited by PTX (but is still abolished by U73122), suggesting the additional involvement of G_q -proteins [21]. However, it should be noted that not all the actions of LPA on $[\text{Ca}^{2+}]_i$ are via $\text{Ins}(1,4,5)\text{P}_3$ production. Thus, in Jurkat T cells, LPA does not stimulate $\text{Ins}(1,4,5)\text{P}_3$ production, and Ca^{2+} mobilization is not from an $\text{Ins}(1,4,5)\text{P}_3$ -sensitive store [7]. Therefore, the possibility remains for the existence of other LPA receptors with potentially novel signalling mechanisms.

In the present study we have investigated LPA-induced Ca^{2+} signalling in human SH-SY5Y neuroblastoma cells. We report that in SH-SY5Y cells, activation of an endogenous LPA receptor leads to Ca^{2+} mobilization, independent of the phosphoinositide signalling pathway, but dependent on the action of sphingosine

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; CLB, cytosol-like buffer; NAS, *N*-acetylsphingosine; dihydro-NAS, dihydro-*N*-acetylsphingosine; DMS, *N,N*-dimethylsphingosine; FAF-BSA, fatty acid-free BSA; fura-2/AM, fura-2 acetoxymethyl ester; GDP[S], guanosine 5'-[γ -thio]-diphosphate; GPCR, G-protein-coupled receptor; InsP_3R , $\text{Ins}(1,4,5)\text{P}_3$ receptor; KHB, Krebs–Henseleit buffer; LPA, lysophosphatidic acid (1-acyl-*sn*-glycerol-3-phosphate); MCh, methacholine; PLC, phosphoinositide-specific phospholipase C; PTX, pertussis toxin; RyR, ryanodine receptor; SPP, sphingosine 1-phosphate.

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kinase, and thus represents a novel signalling pathway for LPA. Comparisons with an established $\text{Ins}(1,4,5)\text{P}_3$ -mediated, Ca^{2+} -mobilizing receptor system have been made using methacholine (MCh) to stimulate endogenous muscarinic M_3 -receptors in SH-SY5Y cells.

EXPERIMENTAL

Materials

ATP, anti-rabbit IgG antiserum–horseradish peroxidase conjugate, β -escin, EGTA, fatty acid-free BSA (FAF-BSA), ionomycin, low-molecular-mass heparin (4–6 kDa), LPA (1-oleoyl-*sn*-glycerol-3-phosphate), PTX and phosphatidic acid (1,2-diacyl-*sn*-glycerol-3-phosphate) were from Sigma. Fura-2 acetoxy-methyl ester (fura-2/AM), fura-2 free-acid and fluo-3 free-acid were from Molecular Probes. *myo*-[^3H]Inositol (117 Ci/mmol), [^3H]Ins(1,4,5) P_3 (58.4 Ci/mmol) and enhanced chemiluminescence (ECL) reagents were from Amersham. *N*-Acetylsphingosine (NAS), dihydro-NAS, *N,N*-dimethylsphingosine (DMS) and ionomycin (free acid) were from Calbiochem. Ins(1,4,5) P_3 was obtained from CellSignal and sphingosine 1-phosphate (SPP) from Biomol. For experiments utilizing permeabilized cells, reagents were made up in plasticware and pretreated with Chelex beads (Bio-Rad) to remove contaminating Ca^{2+} .

Cell culture

SH-SY5Y neuroblastoma cells (passage 70–90) were grown in minimal essential medium with Earle's salts, 10% (v/v) fetal calf serum, 2 mM L-glutamine, 2.5 $\mu\text{g}/\text{ml}$ fungizone, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37 °C in a humidified atmosphere of 5% $\text{CO}_2/95\%$ air.

Measurement of Ins(1,4,5) P_3 mass and total [^3H]inositol phosphates

SH-SY5Y neuroblastoma cells were grown to confluency on 24-well plates. Incubations were performed in Krebs–Henseleit buffer (KHB: 120 mM NaCl/4.8 mM KCl/1.2 mM MgSO_4 /2 mM CaCl_2 /1.2 mM KH_2PO_4 /25 mM NaHCO_3 /5 mM Hepes, pH 7.2) at 37 °C. For measurement of total inositol phosphates, cells were pretreated with KHB supplemented with 10 mM LiCl for 10 min to block inositol monophosphatase activity. Cellular Ins(1,4,5) P_3 mass and total InsP levels were then determined as previously described [23].

Measurement of $[\text{Ca}^{2+}]_i$ in cell suspensions

Confluent monolayers of SH-SY5Y neuroblastoma cells grown in 175 cm^2 flasks were harvested by incubation in 0.9% (w/v) NaCl, 0.02% (w/v) EGTA and 10 mM Hepes, pH 7.2. Cells were washed twice with KHB, resuspended to a density of approx. 1×10^6 cells/ml, and then incubated with 3 μM fura-2/AM for 45 min at room temperature. Aliquots ($\sim 0.5 \times 10^6$ cells) of this suspension were washed once, then resuspended in 2 ml of KHB in a stirred fluorimetry cuvette at 37 °C. Fluorescence was measured with a Perkin-Elmer LS-50B luminescence spectrometer (alternate excitation wavelengths of 340 nm and 380 nm; emission wavelength, 505 nm). At the end of each experiment, R_{max} (the maximum ratio of emission intensities after excitation at 340 and 380 nm) was determined by the addition of 0.1% Triton X-100, then R_{min} was estimated using 4 mM EGTA. Autofluorescence values, determined in parallel experiments using an aliquot of each cell suspension

before the addition of fura-2/AM, were subtracted from fluorescence intensities before calculation of $[\text{Ca}^{2+}]_i$. Ratios of emission intensities resulting from excitation at 340 and 380 nm were converted into $[\text{Ca}^{2+}]_i$ values using the method of Grynkiewicz et al. [24], taking the affinity of fura-2 for Ca^{2+} as 224 nM at 37 °C. For nominally Ca^{2+} -free conditions, CaCl_2 was omitted from the KHB ($[\text{Ca}^{2+}] < 5 \mu\text{M}$ measured using 1 μM fluo-free acid). CaCl_2 (2 mM) was then added before the addition of Triton X-100, in order to determine R_{max} .

Measurement of $[\text{Ca}^{2+}]_i$ in permeabilized cells

Confluent monolayers of SH-SY5Y neuroblastoma cells were harvested as described above and then washed twice in cytosol-like buffer (CLB: 140 mM KCl/5 mM NaCl/2.4 mM MgCl_2 .6 H_2O /2 mM ATPNa_2 /5 mM $(\text{CH}_3\text{COONa})_2$.6 H_2O /10 mM Hepes, pH 7.2 at 37 °C). The cells were resuspended in 2 ml of CLB ($[\text{Ca}^{2+}]$ buffered to 100–200 nM using EGTA) containing 1 μM fluo-3-free acid in a stirred cuvette at 37 °C. Each experimental condition was examined in series using CLB containing the same amount of EGTA. Fluorescence intensities were measured continuously using a Perkin-Elmer LS-50B luminescence spectrometer with an excitation wavelength of 505 nm and an emission wavelength of 530 nm. Cells were treated with 25 $\mu\text{g}/\text{ml}$ β -escin, resulting in more than 95% of cells becoming permeabilized, as assessed by failure to exclude Trypan Blue. An ATP-regenerating system, consisting of 5 units/ml creatine kinase and 10 mM phosphocreatine, was added directly to the cuvette. Once a stable baseline was attained, test substances were added at appropriate time points, followed by 5 μM ionomycin, then 2 mM CaCl_2 and 0.1% Triton X-100 to estimate the maximum fluorescence, F_{max} , and finally 4 mM EGTA to measure F_{min} . Further addition of EGTA did not lower $[\text{Ca}^{2+}]_i$, suggesting that F_{min} had been achieved.

Single-cell Ca^{2+} imaging

SH-SY5Y cells, grown on 22-mm diam. coverslips (Chance Propeller Ltd., Warley, West Midlands, U.K.) were incubated in KHB supplemented with 8 μM fura-2 for 60 min at 22 °C before being mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope and heated to 37 °C. Agonists were added directly to the perfusion chamber. Images at wavelengths above 510 nm were collected, after excitation at 340 and 380 nm, with an intensified charge-coupled device camera (Photonic Science) using a Quanticell 700 (Applied Imaging) system. Ratiometric values were converted into approximate $[\text{Ca}^{2+}]_i$ as described previously [25]. For experiments concerning the actions of DMS, SH-SY5Y cells were grown to a confluent monolayer on 22 mm coverslips and imaged as described above. Cells were preincubated in KHB to which no Ca^{2+} had been added plus inhibitors (where appropriate) for 10 min before agonist addition. Each condition was directly compared with control responses measured in parallel.

Quenching of fura-2 fluorescence with Mn^{2+}

SH-SY5Y cells, prepared as described above, were pretreated with 250 μM Mn^{2+} in Ca^{2+} -free Hepes buffer (120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 1.6 mM MgCl_2 , 25 mM Hepes and 11 mM D-glucose) for 100 s before addition of agonists in the same buffer. Experiments were conducted at room temperature (~ 22 °C). Fluorescent emissions above 510 nm after excitation at 360 nm (the isosbestic point of fura-2) were measured on a photomultiplier system (Photon Technology International, Sutton, U.K.). The rate of quenching of fura-2 fluorescence by

Mn^{2+} was calculated by a best-fit monoexponential using GraphPad Prism software.

Preparation of cell membranes and immunoblotting

These techniques were performed as described previously [26]. A 1:1000 dilution of polyclonal antiserum CT-1, raised against a synthetic peptide corresponding to the C-terminal 19 amino acid residues of the type 1 InsP_3 receptor (InsP_3R), was used to assess the density of this protein in membranes prepared from vehicle-, MCh- and MCh plus atropine-stimulated cells.

RESULTS

LPA and MCh elevate $[\text{Ca}^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores

LPA, at a supramaximal concentration of $10\text{ }\mu\text{M}$, elevated $[\text{Ca}^{2+}]_i$ by $500 \pm 45\text{ nM}$ ($n = 4$) above a resting $[\text{Ca}^{2+}]_i$ of $108 \pm 4\text{ nM}$ ($n = 24$) within 20 s of addition to intact fura-2-loaded SH-SY5Y cell suspensions (Figure 1A). This increase in $[\text{Ca}^{2+}]_i$ was followed by a return to near resting levels within 3 min of addition. Supramaximal doses of MCh (1 mM) resulted in a peak $[\text{Ca}^{2+}]_i$ of similar magnitude ($576 \pm 35\text{ nM}$ above resting levels, $n = 4$) to that triggered by LPA, although with MCh there was a pro-

Figure 1 LPA- and MCh-evoked $[\text{Ca}^{2+}]_i$ responses in SH-SY5Y cell suspensions

(A) Fura-2-loaded SH-SY5Y cell suspensions were challenged first with a supramaximal dose of LPA ($10\text{ }\mu\text{M}$, first arrow) then with a supramaximal dose of MCh (1 mM, second arrow). The trace shown is representative of four similar experiments. (B) The concentration-dependent effect of LPA on $[\text{Ca}^{2+}]_i$ for SH-SY5Y cell suspensions in KHB (■, 2 mM extracellular $[\text{Ca}^{2+}]$), or nominally Ca^{2+} -free KHB (▲, extracellular $[\text{Ca}^{2+}] < 5\text{ }\mu\text{M}$). Results are means $\pm$ S.E.M. for four separate experiments.

Figure 2 Effect of LPA and MCh on $\text{Ins}(1,4,5)\text{P}_3$ production in SH-SY5Y cells

SH-SY5Y cell monolayers were incubated with KHB for 30 min before addition of LPA ($10\text{ }\mu\text{M}$, ○), MCh (1 mM, □), LPA + MCh (●) or vehicle (■). Incubations were stopped by the addition of 1 M trichloroacetic acid and $\text{Ins}(1,4,5)\text{P}_3$ was assayed in neutralized extracts as described in the Experimental section. Results are means $\pm$ S.E.M. for at least three experiments performed in duplicate.

nounced, and sustained, plateau phase of $206 \pm 13\text{ nM}$ ($n = 4$) above basal $[\text{Ca}^{2+}]_i$ (Figure 1A). The elevation of $[\text{Ca}^{2+}]_i$ by LPA in SH-SY5Y cells was concentration-dependent, with a log EC_{50} of -7.91 ± 0.12 (12.3 nM ; Figure 1B). Responses to LPA were dependent upon the presence of FAF-BSA ($10\text{ }\mu\text{g}$ protein/ml final concentration), without which $[\text{Ca}^{2+}]_i$ increases were half-maximal at micromolar rather than nanomolar concentrations of this phospholipid. FAF-BSA itself caused a slight increase in $[\text{Ca}^{2+}]_i$ ($12 \pm 6\text{ nM}$ above resting $[\text{Ca}^{2+}]_i$, $n = 24$), which was subtracted from all Ca^{2+} traces.

Both the MCh- and LPA-induced increases in $[\text{Ca}^{2+}]_i$ resulted predominantly from mobilization of intracellular Ca^{2+} stores. Under nominally Ca^{2+} -free conditions, LPA increased $[\text{Ca}^{2+}]_i$ with a similar potency [$\log \text{EC}_{50} = -7.80 \pm 0.18$ (15.8 nM , $n = 3$)] and maximal effect ($485 \pm 50\text{ nM}$ above resting $[\text{Ca}^{2+}]_i$, $n = 3$) to control conditions (Figure 1B). Treatment of SH-SY5Y cell suspensions with the sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase pump inhibitor thapsigargin (500 nM) resulted in a slow increase in $[\text{Ca}^{2+}]_i$, followed by a sustained plateau. Supramaximal doses of LPA ($10\text{ }\mu\text{M}$) or MCh (1 mM) failed to elevate $[\text{Ca}^{2+}]_i$ above this thapsigargin-induced plateau, indicating that their effects are dependent on Ca^{2+} mobilization from these intracellular stores (results not shown).

Effect of LPA and MCh on InsP production in SH-SY5Y cells

A supramaximal dose of MCh (1 mM) elicited a biphasic increase in $\text{Ins}(1,4,5)\text{P}_3$ mass levels in SH-SY5Y cells, with a peak at 20 s followed by a sustained plateau (Figure 2). In contrast, a supramaximal dose of LPA ($10\text{ }\mu\text{M}$) failed to increase $\text{Ins}(1,4,5)\text{P}_3$ mass above basal levels (Figure 2). This lack of detectable $\text{Ins}(1,4,5)\text{P}_3$ accumulation was not a consequence of LPA interfering with the mass assay employed, since responses to MCh in the additional presence of LPA were indistinguishable from those to MCh alone (Figure 2). LPA also failed to stimulate InsP production ($3 \pm 12\%$ increase above basal $[\text{InsP}]$ accumulation of $138675 \pm 12100\text{ d.p.m.}$, $n = 3$), as measured over a 20 min incubation with SH-SY5Y cells pretreated with 10 mM Li^+ . Under these conditions, MCh caused a 5.27 ± 0.5 -fold increase in $[\text{InsP}]$ accumulation ($n = 3$).

Figure 3 LPA-evoked $[\text{Ca}^{2+}]_i$ responses are not altered by down-regulation of the InsP_3R in SH-SY5Y cells

Chronic stimulation of SH-SY5Y cells with a supramaximal concentration of MCh (1 mM, 18 h) leads to a reduction in the abundance of the type 1 InsP_3R (A). This was assessed by immunoblot of membranes (20 μg protein/lane) from control (lane 1), MCh- (lane 2) and MCh plus atropine- (lane 3) treated cells using an antiserum which specifically recognizes the type 1 InsP_3R , as described in the Experimental section. Lane 4 was loaded with 2 μg of porcine whole brain membrane protein as a positive control. Molecular-mass values of standards are indicated on the right of the blot. Results are representative of three experiments. (B) $[\text{Ca}^{2+}]_i$ responses to LPA were assessed in SH-SY5Y cell suspensions pre-treated with MCh (●) or MCh plus atropine (○). Results are means $\pm$ S.E.M. for three experiments. Best-fit maximal responses and EC_{50} values were not significantly different between the treatments.

Effect of InsP_3R down-regulation on LPA-induced Ca^{2+} mobilization in SH-SY5Y cells

SH-SY5Y neuroblastoma cells express an InsP_3R Ca^{2+} -release-channel population consisting predominantly of the type 1 isoform [27]. These InsP_3R proteins are markedly down-regulated by chronically stimulating SH-SY5Y cells with muscarinic agonists, such as MCh, resulting in a complete loss of $[\text{Ca}^{2+}]_i$ increases stimulated by muscarinic agonists and a partial reduction in responses to exogenous $\text{Ins}(1,4,5)\text{P}_3$ [28]. Down-regulation of InsP_3Rs was used to examine their role in LPA-mediated Ca^{2+} signalling in SH-SY5Y neuroblastoma cells. Untreated SH-SY5Y cells expressed an anti-type 1 InsP_3R antiserum immunoreactive protein (Figure 3A, lane 1) of similar electrophoretic mobility to that in porcine brain microsomes (lane 4). Stimulation of SH-SY5Y cells for 18 h with 1 mM MCh resulted in a considerable loss of this InsP_3R protein (lane 2). This loss was blocked by co-incubation with 10 μM atropine, a muscarinic antagonist (lane 3), indicating that the effect was receptor-mediated. However, reduction of InsP_3R protein levels had no detectable effect on LPA-mediated Ca^{2+} signalling in fura-2-loaded SH-SY5Y cells. Thus, neither the maximal LPA responses, nor the best-fit EC_{50} values for LPA-induced Ca^{2+} -

Figure 4 Elevations in $[\text{Ca}^{2+}]_i$ elicited by LPA are abolished by GDP[S], but not by heparin, in β -escin-permeabilized SH-SY5Y cells

SH-SY5Y cells were permeabilized using β -escin in CLB, and pre-incubated for 5 min in the presence of vehicle alone (A), 300 μM GDP[S] (B), or 100 $\mu\text{g}/\text{ml}$ low-molecular-mass heparin (C). Cells were subsequently challenged with supramaximal concentrations of LPA (10 μM , first arrow), MCh (1 mM, second arrow), then InsP_3 (5 μM , third arrow). Changes in $[\text{Ca}^{2+}]_i$ were estimated using fluo-3 fluorimetry. The traces shown are representative of at least four experiments giving similar results.

mobilization were significantly different between MCh- and MCh plus atropine-pretreated cells (Figure 3B) ($n = 4$).

Effect of GDP[S] and heparin on Ca^{2+} -mobilization in permeabilized SH-SY5Y cells

In β -escin-permeabilized cells, Ca^{2+} signalling mediated by MCh or LPA, monitored using fluo-3, was well maintained. In control experiments, a supramaximal dose of LPA (10 μM) elevated $[\text{Ca}^{2+}]_i$ from a resting level of 142 ± 8 nM to 458 ± 27 nM, which gradually returned to near resting levels ($n = 5$) (Figure 4A). Subsequent addition of a supramaximal dose of MCh (1 mM) increased $[\text{Ca}^{2+}]_i$ to a peak of 624 ± 48 nM, whereas application of $\text{Ins}(1,4,5)\text{P}_3$ (5 μM) elevated $[\text{Ca}^{2+}]_i$ to 751 ± 74 nM (Figure 4A). Pre-incubation of permeabilized SH-SY5Y cells with 300 μM GDP[S] (5 min) reduced the peak $[\text{Ca}^{2+}]_i$ increase evoked by LPA to 154 ± 29 nM ($P < 0.001$ from control, Student's t -test). Responses to MCh were also reduced by GDP[S] pre-treatment, with a peak elevation in $[\text{Ca}^{2+}]_i$ of 429 ± 60 nM ($P < 0.01$). However, Ca^{2+} mobilization in response to exogenous $\text{Ins}(1,4,5)\text{P}_3$ was unaffected by GDP[S] treatment (Figure 4B) ($n = 4$). Resting $[\text{Ca}^{2+}]_i$ after GDP[S] treatment was 101 ± 14 nM ($n = 4$). Pre-incubation of permeabilized SH-SY5Y cells with

Table 1 Effects of various inhibitors on LPA-induced $[\text{Ca}^{2+}]_i$ increases in intact SH-SY5Y cells

SH-SY5Y cells were pre-incubated with various inhibitors and the effect of these on the $[\text{Ca}^{2+}]_i$ response elicited by LPA was assessed by fura-2 microfluorimetry of cell-suspensions. Results are presented as means $\pm$ S.E.M. of n experiments. Where less than three experiments were carried out, individual values are given in parentheses. Where EC_{50} values were not determined (N.D.), the maximal values given are responses to a supramaximal dose of LPA ($10 \mu\text{M}$). * Significant difference from control response ($P < 0.05$).

Pretreatment	Maximal response {increase in $[\text{Ca}^{2+}]_i$ over basal (nM)}	log EC_{50} (log M)
Vehicle (0.1% DMSO)	464 ± 34 ($n = 4$)	-7.83 ± 0.16 (14.7 nM)
PTX (100 ng/ml, 18 h)	482 ± 21 ($n = 3$)	-7.90 ± 0.10 (12.6 nM)
Ryanodine (10 μM , 30 min)	526 ± 39 ($n = 3$)	N.D.
Procaine (1 mM, 5 min)	510 ± 37 ($n = 3$)	-7.89 ± 0.16 (13.0 nM)
N^{ω} -Nitro-L-arginine (50 μM , 60 min)	492 ± 45 ($n = 3$)	-7.85 ± 0.20 (14.2 nM)
Phorbol 12,13-dibutyrate (1 μM , 5 min)	$211 \pm 19^*$ ($n = 3$)	-7.69 ± 0.24 (20.3 nM)
Bisindolylmaleimide 1 (100 nM, 5 min)	491 (472, 511)	N.D.
H-89 (10 μM , 5 min)	506 ± 31 ($n = 3$)	N.D.
Genistein (100 μM , 5 min)	461 ± 18 ($n = 3$)	N.D.

100 $\mu\text{g}/\text{ml}$ of low-molecular-mass heparin for 5 min resulted in a small decrease in resting $[\text{Ca}^{2+}]_i$ to 115 ± 10 nM ($n = 4$). Heparin pre-treatment dramatically inhibited the Ca^{2+} responses of these cells to MCh (168 ± 19 nM, $P < 0.001$ compared with control) and to $\text{Ins}(1,4,5)\text{P}_3$ (189 ± 17 nM, $P < 0.001$) (Figure 4C). However, $[\text{Ca}^{2+}]_i$ elevation caused by LPA was not significantly altered in the presence of heparin (366 ± 55 nM, $P > 0.05$ versus control).

Effect of signalling-pathway inhibitors on LPA-stimulated Ca^{2+} -mobilization

The results described so far indicate that LPA-induced increases in $[\text{Ca}^{2+}]_i$ do not involve either $\text{Ins}(1,4,5)\text{P}_3$ production or the InsP_3R . Therefore, the effects of a number of pharmacological inhibitors of various signalling pathways on LPA-stimulated Ca^{2+} mobilization were investigated in an attempt to determine the mechanism involved. As can be seen from Table 1, LPA-stimulated increases in $[\text{Ca}^{2+}]_i$ were unaffected by pretreatment with PTX (100 ng/ml, 18 h), ryanodine (10 μM , 30 min), procaine (1 mM, 5 min) and N^{ω} -nitro-L-arginine (50 μM , 60 min). The LPA response was also unaffected by pretreatment with the protein kinase inhibitors bisindolylmaleimide (100 nM, 5 min), H-89 (10 μM , 5 min) or genistein (100 μM , 5 min). However, the peak $[\text{Ca}^{2+}]_i$ response to 10 μM LPA was reduced by 55 $\pm$ 5 % by a 5 min pretreatment with 1 μM phorbol 12,13-dibutyrate (1 μM , 5 min) ($n = 3$).

LPA and MCh stimulate Ca^{2+} -entry into fura-2-loaded SH-SY5Y cells

As LPA appears to mobilize Ca^{2+} via a novel pathway, the ability of the LPA-accessible Ca^{2+} pool to stimulate store-refilling-mediated Ca^{2+} entry was investigated. This was especially important as previous work, in Jurkat human T-cells, reported that LPA-stimulated Ca^{2+} mobilization does not activate Ca^{2+} entry [7]. To determine Ca^{2+} -entry into SH-SY5Y cells, Ca^{2+} in the Hepes buffer was substituted with 250 μM Mn^{2+} , and the rate of Mn^{2+} entry was measured by the ability of this ion to quench fura-2 fluorescence. Application of 250 μM Mn^{2+} itself caused a time-dependent decrease in fura-2 fluorescence, which approximated to a monoexponential decay curve with a rate constant of $0.18 (\pm 0.01) \times 10^{-4} \text{ s}^{-1}$ ($n = 3$). This rate increased to $4.32 (\pm 0.09) \times 10^{-4} \text{ s}^{-1}$ when the cells were stimulated with 10 μM

Figure 5 Stimulation of Mn^{2+} quenching of fura-2 fluorescence by LPA and MCh in SH-SY5Y cells

Fura-2-loaded SH-SY5Y cells, grown on coverslips, were pretreated with Ca^{2+} -free Hepes buffer containing 250 μM Mn^{2+} for 100 s before the addition of LPA (10 μM), MCh (100 μM) or buffer alone, in the continued presence of Mn^{2+} . The ordinate scale is the relative fluorescent intensity at 510 nm after excitation at 360 nm. The rates of quench, as estimated from a best-fit monoexponential, were $0.18 \pm (0.01) \times 10^{-4} \text{ s}^{-1}$ for Mn^{2+} alone, and $4.32 \pm (0.09) \times 10^{-4} \text{ s}^{-1}$ and $10.13 \pm (0.06) \times 10^{-4} \text{ s}^{-1}$ for LPA and MCh respectively. Traces are representative of three experiments.

LPA ($n = 3$) (Figure 5). In comparison, MCh (100 μM) increased the rate of quench to $10.13 (\pm 0.06) \times 10^{-4} \text{ s}^{-1}$ ($n = 3$) (Figure 5).

LPA- and MCh-stimulated Ca^{2+} mobilization in single SH-SY5Y cells

To examine spatiotemporal aspects of the LPA-stimulated Ca^{2+} release pathway, single, fura-2-loaded SH-SY5Y cells were fluorescently imaged. In this set of experiments, LPA (10 μM , 30 s application) stimulated a 448 ± 20 nM increase in basal $[\text{Ca}^{2+}]_i$ (mean data from 19 cells). Basal $[\text{Ca}^{2+}]_i$ was 103 ± 3 nM. After a 5 min wash period with KHB, MCh (10 μM) stimulated a 514 ± 16 nM increase in the same cells. Comparisons of the effects of LPA and MCh on the same cells indicated that although responses originated in the same areas of the cells (near the 'neurite' projections), the rate of increase in $[\text{Ca}^{2+}]_i$, measured as $178 \pm 12 \text{ nM} \cdot \text{s}^{-1}$, was $49 \pm 5\%$ slower in the LPA response (mean data from 19 cells, $P < 0.001$ versus MCh response). These results were essentially the same when the application of agonists was reversed (results not shown).

Figure 6 Effect of DMS on LPA-, MCh- and ionomycin-induced Ca^{2+} mobilization in SH-SY5Y cells

Fura-2-loaded SH-SY5Y cells, grown to confluence on coverslips, were preincubated in Ca^{2+} -free KHB for 10 min in the additional presence of DMS (30 μM) or NAS (30 μM) where indicated. Cells were then stimulated, in the continued absence of Ca^{2+} , with 1 μM LPA (A, B, and C), or 1 mM MCh (D, E and F) or 2 μM ionomycin (G and H). Traces are representative of three separate experiments where each condition was paired with a control response.

Effect of LPA analogues on $[\text{Ca}^{2+}]_i$ in SH-SY5Y cells

Phosphatidic acid and SPP, compounds which share structural similarities with LPA, also elevated $[\text{Ca}^{2+}]_i$ in SH-SY5Y neuroblastoma cells, though with much smaller maximal effects. Thus, SPP (10 μM) and phosphatidic acid (10 μM) increased $[\text{Ca}^{2+}]_i$ by 164 ± 8 nM and 176 ± 8 nM, respectively ($n = 4$). Similar to the LPA response, SPP did not stimulate measurable $[\text{H}^3]\text{InsP}$ formation over a 20 min incubation in the presence of 10 mM Li^+ (138675 ± 12100 and 104625 ± 19175 d.p.m./mg of protein, in the absence and presence of 10 μM SPP respectively) ($n = 3$).

Effect of DMS on LPA-, MCh- and ionomycin-stimulated Ca^{2+} mobilization in SH-SY5Y cells

As LPA-induced Ca^{2+} mobilization did not appear to involve either of the well-characterized pathways of release, we investigated the potential role of sphingosine kinase in SH-SY5Y cells. Previous work has described the ability of SPP, produced from sphingosine via sphingosine kinase in response to activation of GPCRs, to stimulate Ca^{2+} release from intracellular stores [29]. In order to minimize the effects of the sphingosine kinase inhibitor DMS on Ca^{2+} entry [30], experiments were performed in nominally Ca^{2+} -free KHB (see Experimental section). Pre-treatment of SH-SY5Y cells with 30 μM DMS for 10 min slightly increased basal $[\text{Ca}^{2+}]_i$ from 62 ± 2 nM to 70 ± 2 nM ($n = 9$), and reduced the peak Ca^{2+} response to 1 μM LPA by $88 \pm 4\%$ ($P < 0.05$, unpaired t -test; $n = 3$) (Figures 6A and 6B). A similar degree of inhibition block was observed if total Ca^{2+} release, calculated as the area under the curve, was measured ($87 \pm 4\%$

inhibition). Preincubation with 30 μM DMS also reduced the Ca^{2+} response to 1 mM MCh ($57 \pm 11\%$ reduction in peak and $41 \pm 12\%$ reduction in total Ca^{2+} release; $P < 0.05$, $n = 3$) (Figures 6D and 6E). In contrast, preincubation with either 30 μM NAS (Figures 6C and 6F), or the bio-inactive dihydro-NAS (results not shown), compounds which do not inhibit sphingosine kinase, did not block the Ca^{2+} responses to LPA or MCh ($n = 3$). DMS (30 μM) also reduced the peak Ca^{2+} response to the addition of 2 μM ionomycin by $27 \pm 11\%$ ($n = 3$) (Figures 6G and 6H). However, this was not significantly different from the control values, and if the action of DMS was measured on total Ca^{2+} release no effect was observed ($2 \pm 14\%$ decrease).

DISCUSSION

There is growing evidence in the literature that LPA, and structural analogues such as SPP, represent a novel family of signalling molecules which are responsible for a diverse array of cellular responses [3]. The search for the cell-surface receptors responsible for initiating the intracellular responses to these signalling molecules has led to the discovery of Edg-2 and Edg-4, putative GPCRs for LPA [14,15], and Edg-1, a putative GPCR for SPP [31]. However, it is currently unclear which of the cloned receptor types are responsible for the multiple cellular responses observed in the various cell types, and it seems probable that more LPA receptors remain to be discovered. In the present study, investigating the effects of LPA on Ca^{2+} signalling in human SY-SY5Y neuroblastoma cells, we have obtained evidence for an LPA receptor which is mechanistically distinct from

those reported in many other cell types. In addition, in SH-SY5Y cells LPA appears to utilize a Ca^{2+} release pathway different from those previously described.

Similar to effects in other cell types, the addition of LPA to SH-SY5Y cells produces a rapid Ca^{2+} mobilization response. Both LPA and the muscarinic acetylcholine receptor agonist MCh release intracellular Ca^{2+} from thapsigargin-sensitive stores within these cells. However, unlike MCh, which rapidly elevates $\text{Ins}(1,4,5)\text{P}_3$ levels and stimulates $[\text{H}]\text{InsP}$ accumulation in Li^+ -treated SH-SY5Y cells, LPA does not cause any detectable activation of PLC activity. There remains the possibility that LPA could cause very localized elevations in $\text{Ins}(1,4,5)\text{P}_3$ levels, not detectable by current methods, which are sufficient to activate InsP_3Rs in the immediate vicinity of the LPA receptor [25,32]. However, this appears unlikely, as down-regulation of InsP_3R proteins in SH-SY5Y cells had no significant effect on the Ca^{2+} -mobilization concentration-response relationship for LPA. Furthermore, the InsP_3R antagonist heparin, which blocks Ca^{2+} release in response to MCh and exogenously applied $\text{Ins}(1,4,5)\text{P}_3$ in permeabilized SH-SY5Y cells, does not affect the response to LPA. These observations are further supported by recent preliminary experiments using a $[\text{S}]\text{guanosine } 5'\text{-}[\gamma\text{-thio}]\text{-triphosphate-binding immunoprecipitation strategy}$ [33], which demonstrated that in SH-SY5Y cells, LPA-receptor activation failed to stimulate $\text{G}_{q/11}\alpha$ activity, whereas M3 muscarinic receptor activation produced a robust response (K. W. Young and S. R. Nahorski, unpublished work).

These observations indicate that, unlike the cloned Edg-2 and Edg-4 receptors, the LPA receptor in SH-SY5Y cells does not utilize the phosphoinositide signalling pathway. In skeletal and cardiac muscles, the predominant mechanism of Ca^{2+} release from intracellular stores is via the ryanodine receptor (RyR) [34]. RyRs are present throughout the mammalian nervous system [34], and ryanodine-sensitive Ca^{2+} pools coupled to extracellular stimuli have been characterized in a number of mammalian cell-lines, including HeLa [35], PC-12 [36] and SK-N-BE [37] cells. Furthermore, the presence of both the type 2 (cardiac type) RyR protein and Ca^{2+} pools sensitive to caffeine, an agonist of these channels, in SH-SY5Y cells have been demonstrated recently [27]. However, LPA does not appear to activate RyRs in SH-SY5Y cells, as Ca^{2+} mobilization was unaffected by ryanodine itself, or the RyR-antagonist procaine. Modulation of ryanodine-sensitive Ca^{2+} stores by the nitric oxide/cGMP pathway, via cADP-ribose, has recently been characterized in neurosecretory PC-12 cells [38]. However, LPA-induced Ca^{2+} mobilization was unaffected by N^{ω} -nitro-L-arginine, a nitric oxide synthase inhibitor. Interestingly, procaine inhibits Ca^{2+} -activated Cl^- currents elicited by LPA in *Xenopus* oocytes [39].

A third intracellular Ca^{2+} -release pathway, which involves sphingolipids, has recently been described [40–43], and although the pathway remains to be fully elucidated, a novel intracellular sphingolipid-gated Ca^{2+} -channel (termed SCAmPER) has been cloned [44]. Furthermore, agonists acting on GPCRs have been shown to stimulate Ca^{2+} mobilization through the production of SPP [29], an action that occurs via the enzyme sphingosine kinase. In the present study, DMS, a competitive inhibitor of sphingosine kinase [45], almost completely blocked the Ca^{2+} response to a supramaximal application of LPA and approximately halved the peak Ca^{2+} response to MCh. This was not a non-specific effect of sphingolipids, as the related compounds NAS and dihydro-NAS were without effect. Similarly, the effect of DMS was not due to an emptying of the intracellular Ca^{2+} store, as there was no significant reduction in the ionomycin-sensitive Ca^{2+} pool. Although the competitive nature of the DMS inhibition makes interpreting these findings more complicated,

the results suggest that both LPA and MCh utilize the production of SPP, although to different extents. Thus LPA, which does not stimulate $\text{Ins}(1,4,5)\text{P}_3$ production in SH-SY5Y cells, may release Ca^{2+} solely by increasing intracellular SPP. In contrast, MCh, which produces a robust $\text{Ins}(1,4,5)\text{P}_3$ response, is only partially sensitive to DMS, and so may be able to utilize dual pathways leading to Ca^{2+} release. Previously, muscarinic M_2 - and M_3 -receptors transfected into HEK-293 cells have been shown to mobilize Ca^{2+} through the production of intracellular SPP [29], and so this may be a common pathway for this receptor type, although in HEK-293 cells M_2 - and M_3 -receptors also stimulate $\text{Ins}(1,4,5)\text{P}_3$ production. Importantly, in HEK-293 cells, DMS did not alter muscarinic receptor-induced InsP accumulation, or the ability of $\text{Ins}(1,4,5)\text{P}_3$ to release Ca^{2+} directly [29], indicating that DMS did not interfere with the phosphoinositide signalling pathway. Similarly in SH-SY5Y cells, DMS did not inhibit MCh-induced InsP production (results not shown).

The actions of LPA in SH-SY5Y cells may therefore prove to be a useful model for investigating the role of sphingosine kinase activation in Ca^{2+} -mobilizing responses, without the additional $\text{Ins}(1,4,5)\text{P}_3$ -sensitive component that is present with other GPCRs. That the LPA response involves a GPCR is strongly suggested by the sensitivity of LPA-mediated Ca^{2+} signalling, in permeabilized SH-SY5Y cells, to GDP[S] . The lack of effect of PTX pretreatment excludes an involvement of G_i -proteins and distinguishes responses observed in the present study from those mediated by the phosphoinositide-linked recombinant Edg-2 and Edg-4 receptors. LPA has recently been shown to be a low-affinity agonist for the cell surface SPP-receptor, Edg-1 [46]. However, in the present study it is unlikely that LPA-responses occurred via this receptor, as Edg-1 effects are also G_i -mediated [47], and extracellular SPP itself only produces a small Ca^{2+} response in intact SH-SY5Y cells. The Ca^{2+} response to LPA was inhibited by short-term pretreatment with phorbol 12,13-dibutyrate, suggesting some involvement of protein kinase C. Inhibition of GPCR-mediated responses by phorbol ester treatment has been reported previously [48], and may indicate that phosphorylation of the LPA receptor attenuates signalling. However, effects on elements downstream of receptor activation cannot be excluded. In addition, the lack of effect of the kinase inhibitors bisindolylmaleimide, H-89 and genistein on LPA-induced Ca^{2+} mobilization indicates that it is unlikely that the LPA-receptor utilizes activation of C-, A- or tyrosine-kinases in the Ca^{2+} response. The observation that extracellular SPP itself produces a modest increase in $[\text{Ca}^{2+}]_i$ is interesting, as it suggests that this bioactive lipid may have both intracellular and extracellular signalling effects [49]. What is also apparent from this study is that emptying the LPA-, and hence sphingolipid-, sensitive intracellular Ca^{2+} pool stimulates the process of store-refilling-mediated Ca^{2+} entry, as indicated by Mn^{2+} quenching. This would appear to differentiate the LPA response in SH-SY5Y cells from the non- InsP_3R -, non-RyR-mediated effects of LPA in Jurkat T-cells [7].

Perhaps the most remarkable feature of LPA-mediated Ca^{2+} mobilization in SH-SY5Y cells is that, despite the involvement of different signalling pathways, increases in $[\text{Ca}^{2+}]_i$ are almost indistinguishable from those stimulated in these cells by MCh. Thus, without recruiting either InsP_3Rs or RyRs, LPA can trigger a Ca^{2+} increase which is similar in magnitude to, and only marginally slower than, that stimulated by MCh. Furthermore, the similarities between Ca^{2+} -release responses to LPA and MCh suggest that the production of SPP, if indeed this proves to be the LPA-stimulated pathway, may be of equal significance in Ca^{2+} -mobilization responses to the more established $\text{Ins}(1,4,5)\text{P}_3$ pathway. It will be of interest therefore to determine the relative abilities of LPA and MCh to stimulate SPP production in SH-

SY5Y cells. Such studies are presently underway in this laboratory. In the light of the present study, however, the action of LPA in SH-SY5Y cells may prove to be a valuable means of investigating non-Ins(1,4,5) P_3 -mediated Ca^{2+} signalling.

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