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Expression and Function of Lysophosphatidic Acid Receptors in Cultured Rodent Microglial Cells*

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Microglia are the resident tissue macrophages of the central nervous system. They are rapidly activated by a variety of insults; and recently, receptors linked to cytoplasmic Ca^{2+} signals have been implicated in such events. One potential class of receptors are those recognizing lysophosphatidic acid (LPA). LPA is a phospholipid signaling molecule that has been shown to cause multiple cellular responses, including increases in cytoplasmic calcium. We examined whether any of the known LPA receptor genes (*lp_{A1}/Edg2*, *lp_{A2}/Edg4*, and *lp_{A3}/Edg7*) are expressed by cultured mouse or rat microglia. Reverse transcriptase-polymerase chain reaction indicated that mouse microglia predominantly expressed the *lp_{A1}* gene, whereas rat microglia predominantly expressed *lp_{A3}*. Although LPA induced increases in the cytoplasmic Ca^{2+} concentration in both microglial preparations, the responses differed substantially. The Ca^{2+} signal in rat microglia occurred primarily through Ca^{2+} influx via the plasma membrane, whereas the Ca^{2+} signal in mouse microglia was due to release from intracellular stores. Only at high concentrations was an additional influx component recruited. Additionally, LPA induced increased metabolic activity in mouse (but not rat) microglial cells. Our findings provide evidence for functional LPA receptors on microglia. Thus, LPA might play an important role as a mediator of microglial activation in response to central nervous system injury.

Microglia are the resident immune cells of the central nervous system (1). Once activated during a central nervous system insult, microglial cells undergo a rapid, graded response that involves cell migration, proliferation, cytokine release, and trophic and/or cytotoxic effects (1, 2). The signals and mechanisms of microglial activation following central nervous system injury are just beginning to be identified. Although several cytokines, growth factors, and peptides are known to stimulate microglia via receptors on the cell surface (for review, see Ref. 3), other molecules are likely to have important roles as well.

One signaling molecule that might regulate microglial activation during central nervous system injury is lysophosphatidic acid (LPA).[1] LPA is a bioactive phospholipid mediator that is produced by activated platelets, injured cells, and cells stimulated by growth factors (4–7). LPA was initially shown to cause multiple biological effects on fibroblasts, including increases in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) and increased proliferative activity (8, 9). The effects of LPA are mediated through three distinct G-protein-coupled receptors that are encoded by different genes (*lp_{A1}/Edg2*, *lp_{A2}/Edg4*, and *lp_{A3}/Edg7*) (for review, see Refs. 5–7). The three LPA receptors differ somewhat in their signal transduction properties. Although activation of any of the three receptors results in increased $[Ca^{2+}]_c$, only the LP_{A1}-mediated response is completely blocked by pertussis toxin (PTX) (10, 11). All three receptors mediate inhibition of adenylate cyclase, although LP_{A1} is most effective (7, 12). In addition, although LP_{A1} and LP_{A2} receptors mediate acute neurite retraction, the LP_{A3} receptor does not (7, 12).

Recent data suggest that LPA may play a prominent role in the central nervous system. Astrocytes are known to respond to LPA with $[Ca^{2+}]_c$ increases, lipid peroxidation, DNA synthesis, and cell rounding (13–16). Oligodendrocytes respond to LPA with $[Ca^{2+}]_c$ increases (17) and have been shown to express the *lp_{A1}* gene *in vivo* (18). Neuroblasts, which also express the *lp_{A1}* gene, respond to LPA with cell rounding, proliferation, increased chloride conductances, and activation of a nonselective cation conductance (19, 20). Mature neurons respond to LPA with $[Ca^{2+}]_c$ increases, rapid growth cone collapse, and neurite retraction (21, 22). LPA has also been shown to increase the tight junction permeability of cerebral endothelium and to alter cerebrovascular reactivity (23, 24). To date, however, the effects of LPA on microglial cells have not been investigated.

In this study, we show that cultured mouse and rat microglia each express LPA receptor genes, albeit different ones. We show that the receptors possess functional signal transduction properties, as the cells respond to LPA with $[Ca^{2+}]_c$ signals and increased metabolic activity. Although specific signal transduction aspects may differ between species, our results suggest that LPA is an important signaling molecule that might activate microglial cells in response to central nervous system injury.

EXPERIMENTAL PROCEDURES

Preparation of Mouse and Rat Microglial Cells—Microglial cells were prepared from the cortices of newborn (postnatal days 1–3) Swiss-Webster mice or Long-Evans rats as described previously (25, 26). In brief, cortical tissue was freed from blood vessels and meninges. Tissue was minced, trypsinized for 20 min, triturated with a fire-polished

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[1] The abbreviations used are: LPA, lysophosphatidic acid; $[Ca^{2+}]_c$, cytoplasmic Ca^{2+} concentration; PTX, pertussis toxin; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pairs; GFAP, glial fibrillary acidic protein.

pipette, and washed twice. The cortical cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Inc.) and 20% L929 conditioned medium. Cells were kept at 37 °C in a 5% CO_2 and 95% air incubator, and the medium was changed every 3–4 days. After 9–21 days, microglial cells were separated from the underlying astrocytic monolayer by gentle agitation using their differential adhesive properties. The resulting cell suspension was spun down at 200 × *g* for 10 min. The cell pellet was resuspended in Dulbecco's modified Eagle's medium/nutrient mixture F-12 with 10% fetal bovine serum. For imaging experiments, microglial cells were plated on poly-L-lysine-coated glass coverslips at a density of 2×10^4 cells × cm^{-2} and were allowed to settle for 20 min. Non-adhesive cells were removed by washing in Dulbecco's modified Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (Life Technologies, Inc.). Cells were cultured in macrophage serum-free medium supplemented with G5 supplement, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Inc.) and kept in the same medium for 1–2 days before being used for experiments. For RNA isolation, cells were plated in 10-cm polystyrene culture dishes at a density of 5×10^6 and further processed as described above. Cultures routinely consisted of ~98% microglial cells as determined by staining with *Griffonia simplicifolia* isolectin B4 (Vector Labs, Inc., Burlingame, CA). In addition, reverse transcriptase-polymerase chain reaction (RT-PCR) indicated that the microglial cultures had robust CD11b expression (a microglial marker) and no expression of GFAP (an astrocyte marker; see "Molecular Receptor Characterization by RT-PCR" for primers).

The mouse microglial cell line N9 was a kind gift of Dr. M. Righi (International School for Advanced Studies, Trieste, Italy) and cultured in accordance with the original publication (27). The mouse microglial cell line BV2 was a kind gift of Dr. E. Blasi (University of Perugia, Perugia, Italy) and cultured in accordance with the original publication (28).

Molecular Receptor Characterization by RT-PCR—To generate a cDNA template, total RNA from cultured microglial cells was isolated using the RNeasy minikit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. For reverse transcription, a 40-μl reaction mixture consisting of 1× Superscript first-strand synthesis buffer, 20 units of RNasin (Roche Molecular Biochemicals), 0.5 mM each dNTP (Sigma), 10 μg of RNA, 2 μl (200 pmol) of diluted random hexamers (Roche Molecular Biochemicals), and 200 units of Superscript (Life Technologies, Inc.) was incubated at 23 °C for 10 min, 42 °C for 60 min, and 95 °C for 5 min; cooled on ice; diluted to 100 μl with H_2O; and stored at −20 °C. PCRs of 25 μl consisted of 1× PCR buffer (75 mM Tris (pH 9.0) and 15 mM $(NH_4)_2SO_4$), 0.25 mM each dNTP, 0.5 mM each primer, 1 μl of template (*i.e.* the equivalent of 0.1 μg of total RNA), and 0.5 units of *Taq* polymerase (*Taq* polymerase was added after the reaction mixture was overlaid with mineral oil and heated to 90 °C). Reaction mixtures were cycled 35 times at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min. The number of cycles for all PCRs shown was adjusted so that product was not maximized as compared with quantities obtained with excess product as template (35 cycles were used for each LPA receptor gene, and 25 cycles were used for the β-actin reactions). All primers were designed to different exons within genes so genomic DNA contaminants would not be amplified. Primer combinations, relative locations, and expected product sizes were as follows: lp_{A1}e3mh/lp_{A1}e4mh, exons 3 and 4, 349 bp; lp_{A2}e2mh1/lp_{A2}e3mh1, exons 2 and 3, 798 bp; lp_{A3}e1dmh/lp_{A3}e2mh1, exons 2 and 3, 382 bp; GFAPmh3/GFAPmh4, 956 bp; CD11bmh1/CD11bmh2, 545 bp; and β-actin a (any)/β-actin b (any), 630 bp. Primer sequences were as follows: lp_{A1}e3mh1, 5′-TCT TCT GGG CCA TTT TCA AC-3′; lp_{A1}e4mh1, 5′-TGC CTR AAG GTG GCG CTC AT-3′; lp_{A2}e2mh1, 5′-CCT ACC TCT TCC TCA TGT TC-3′; lp_{A2}e3mh1, 5′-TAA AGG GTG GAG TCC ATC AG-3′; lp_{A3}e1dmh, 5′-GGA ATT GCC TCT GCA ACA TCT-3′; lp_{A3}e2mh1, 5′-GAG TAG ATG ATG GGG TTC A-3′; GFAPmh3, 5′-GCT TCC TGG AAC AGC AAA AC-3′; GFAPmh4, 5′-TCC TCT TGA GGT GGC CTT CT-3′; CD11bmh1, 5′-TTT GTC TCA ACT GTG ATG GA-3′; CD11bmh2, 5′-GAG ACA TCT CAT GCT CAA A-3′; β-actin a (mouse), 5′-ACA GCT TCT TTG CAG CTC C-3′; β-actin a (rat), 5′-TAC AAC CTC CTT GCA GCT CC-3′; and β-actin b (mouse/rat), 5′-GGA TCT TCA TGA GGT AGT CTG TC-3′.

$[Ca^{2+}]_c$ Measurements in Cultured Microglial Cells—Microglial cells were loaded with Ca^{2+} indicator by incubation of glass coverslips with adhered cells in HEPES bathing solution supplemented with 5 μM Fura-2 acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR) and 0.02% Pluronic F-127 (Molecular Probes, Inc.) for ~30 min at room temperature. For $[Ca^{2+}]_c$ measurements, microglial cells on coverslips were placed in a perfusion chamber on the stage of an inverted microscope (Diaphot 200, Nikon Inc., Melville, NY) equipped with either 20×/0.75 NA air or 40×/1.3 NA oil immersion objectives. Fura-2 was excited using a Lambda DG-4 filter system (Sutter Instrument Co., Novato, CA) at wavelengths of 340 and 380 nm, and the fluorescence emission was collected at a wavelength of 510 ± 20 nm via a band-pass filter. Acquisition of the fluorescence data and image analysis were performed using a digital imaging system (R3, Inovision Corp., Durham, NC) and standard PC evaluation software. Ratios were collected at time intervals varying between 1 and 5 s. $[Ca^{2+}]_c$ was calculated using the method of Grynkiewicz *et al.* (29). R_{min}, R_{max}, and *b* were obtained using a Fura-2 Ca^{2+} calibration kit (F-6774, Molecular Probes, Inc.). Substances were applied by changing the perfusion buffer. LPA was stored as a 1 mM stock solution in Me_2SO at −20 °C, and LPA-containing buffer was vigorously vortexed immediately prior to each application. Application of Me_2SO alone did not induce any detectable $[Ca^{2+}]_c$ signals. The use of bovine serum albumin-solubilized LPA was not feasible, as the protein interfered with the perfusion system. At the end of an experiment, the application of ATP served as a control for the viability and responsiveness of the cells. ATP is known to cause robust increases in $[Ca^{2+}]_c$ via Ca^{2+} release and influx (30, 31). $[Ca^{2+}]_c$ signals were defined as $[Ca^{2+}]_c$ changes ≥1.2 × base line. Standard PC data analysis software was used for post-imaging analysis. All experiments were carried out with at least three independent cultures. Recordings are presented as population responses of one coverslip unless otherwise indicated.

Assay for Microglial Metabolic Activity/Proliferation—Microglial metabolic activity was assessed in 96-well plates with 2×10^4 cells/well in 150 μl of RPMI 1640 medium (Life Technologies, Inc.). LPA (1, 3, 10, and 30 μM) was directly dissolved in RPMI 1640 medium supplemented with lipid-free bovine serum albumin (0.1%) to improve the solubility of LPA. Controls received the carrier only. 1–2 days after plating, cells were stimulated with LPA for 1 h, carefully washed with Dulbecco's modified Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, and cultured in 150 μl of RPMI 1640 medium. For assessing the metabolic activity 24 and 48 h later, cells were incubated with 15 μl of the cell proliferation test reagent WST-1 (Roche Molecular Biochemicals) for 30–60 min. This colorimetric assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cells. The amount of cleaved WST-1 is proportional to the activity of mitochondria and can be used as a readout for metabolic activity and/or proliferation. The assay was used in accordance with the manufacturer's instructions. Absorption was measured at 450 nm with 750 nm as reference in a microplate reader (EL 340, Bio-Tek Instruments, Winooski,VT). Metabolic activity/proliferation is given as percent change compared with control. All experiments were carried out with a minimum of six wells/condition ($n \geq 6$).

Solutions and Reagents—Standard HEPES bathing solution for experiments with cultured cells was composed of 150 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/NaOH, and 10 mM glucose (pH 7.35). To obtain Ca^{2+}-free solution, $CaCl_2$ was omitted; $MgCl_2$ was increased to 2 mM; and 0.5 mM EGTA was added. For the application of lidocaine (40 mM), NaCl was reduced to 110 mM. Reagents were prepared from stock solutions stored at −20 °C by dilution in standard or calcium-free extracellular bathing solution immediately prior to use. If not stated otherwise, chemicals were obtained from Sigma.

Statistics—Statistical evaluation was carried out using PRISM software (GraphPAD, San Diego, CA). Comparisons were made by analysis of variance with Bonferroni's post-test, and $p < 0.05$ was considered to be significant. Data are given as means ± S.E.

RESULTS

LPA Receptor Gene Expression in Cultured Microglial Cells—To examine expression of the three known LPA receptor genes (lp_{A1}/*Edg2*, lp_{A2}/*Edg4*, and lp_{A3}/*Edg7*) in microglial cells, we utilized RT-PCR (Fig. 1). Primers were designed to sequences conserved between mouse and human sequences. Robust cDNA amplification from rat testes and kidney, two tissues that express all three receptor genes, showed that the primers also recognized rat sequences. In three independent primary microglial cell cultures from mouse, we found that lp_{A1} was the primary LPA receptor gene expressed, with little or no lp_{A2} and lp_{A3}. In contrast, three independent primary microglial cultures from rat expressed primarily lp_{A3}, with little or no lp_{A1} and lp_{A2}. We also examined expression of the three receptors in two microglial cell lines. The mouse N9 cell line

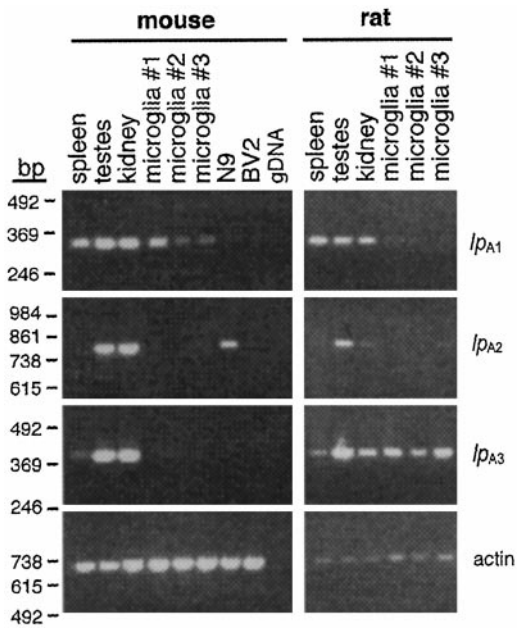

FIG. 1. **RT-PCR detection of LPA receptor genes in microglial cDNA.** The templates used in each RT-PCR are listed above each lane, including tissues known to express each of the transcripts abundantly (testes and kidney) or sparingly (spleen), genomic DNA (*gDNA*), and the N9 and BV2 immortal microglial cell lines. Each *horizontal row* represents amplification with the primers specific to the gene indicated to the right. Three independent microglial preparation samples are shown for both primary mouse and rat microglial cells. Amplification intensity of β-actin was used as a relative loading control for cDNA quantity.

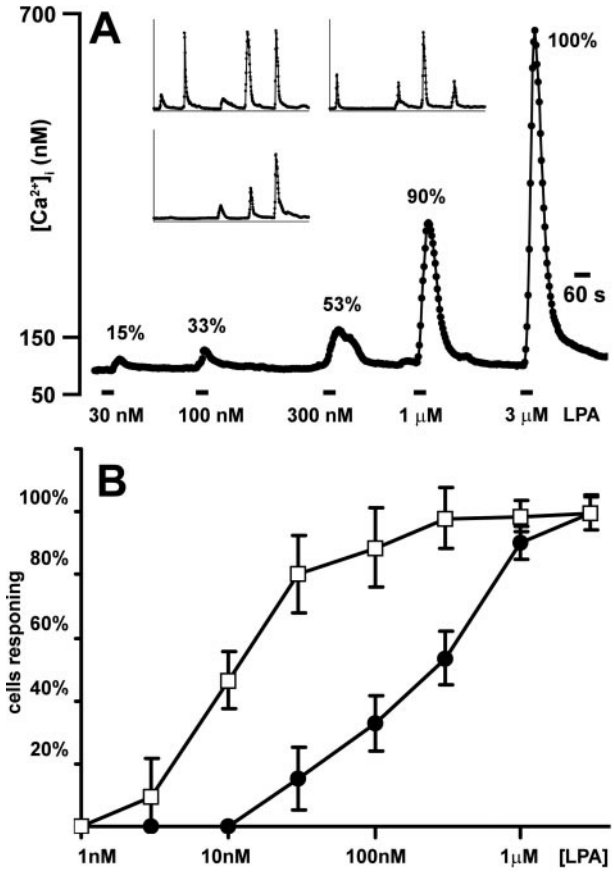

FIG. 2. **LPA-induced $[Ca^{2+}]_c$ signals in cultured rat microglial cells.** *A*, Fura-2-loaded rat microglial cells were perfused with varying concentrations of LPA. Shown is a population response of microglia (n = 73) stimulated with increasing concentrations of LPA (0.03–3 μM). The percentage of cells responding with a $[Ca^{2+}]_c$ signal to LPA stimulation increased in a concentration-dependent fashion. The *inset traces* show the responses of individual cells to the same series of LPA concentrations. Note the high response variability, likely due to poor solubility and micelle formation of LPA in buffer solution. *B*, shown is a comparison of concentration-response relationships for mouse and rat microglial cells. Mouse cells (□) showed a higher sensitivity to LPA then rat microglia (●). Data are given as means ± S.E.

expressed only lp_{A2}, whereas BV2 expressed none of the receptors. These results suggest that microglial cells from mouse and rat would respond to LPA, although these responses might differ based on the distinct coupling properties of the three receptors.

LPA-induced $[Ca^{2+}]_c$ Signals in Rat Microglial Cells—To investigate the ability of LPA to induce $[Ca^{2+}]_c$ signals in cultured microglia, Fura-2-loaded cells were stimulated with varying concentrations of LPA in a perfusion chamber. We initially determined that individual rat microglial cells had variable responses to the same concentrations of LPA, although increasing LPA concentrations resulted in a higher probability of response (Fig. 2*A*, *insets*). This variability at the level of single cells was probably due to the poor solubility of LPA in aqueous solutions and the resulting formation of micelles. To compensate for this, average population responses were determined, which facilitated quantitative comparisons. Microglial cells were stimulated consecutively with 0.03, 0.1, 0.3, 1.0, and 3 μM LPA. In the shown experiment (Fig. 2*A*), the percentage of rat microglial cells responding with a $[Ca^{2+}]_c$ signal (defined as $\geq 1.2\times$ base line) to LPA stimulation increased in a concentration-dependent fashion from 15% for 30 nM LPA to 100% for 3 μM LPA (Fig. 2*A*). No response was seen at 10 nM in any experiment. Pooled data from several experiments were used to construct a concentration-response graph (Fig. 2*B*). Repeated application of LPA (*e.g.* three applications of $\leq$3 μM LPA for 30 s, with 5-min intervals) did not lead to a reduction in the number of cells responding or a significant decrease in the response amplitude (data not shown). Based on these findings, we used 3 μM LPA for our further studies. We did not use higher LPA concentrations, as they would have necessitated increased solvent (Me_2SO) concentrations of >0.3%.

LPA-induced $[Ca^{2+}]_c$ Signals in Mouse Microglial Cells—Mouse microglial cells showed a similar heterogeneity in responses of individual cells (data not shown). Nevertheless, LPA induced $[Ca^{2+}]_c$ signals with two different kinetic profiles. At concentrations $\leq$1 μM, LPA induced a transient $[Ca^{2+}]_c$ signal. At higher concentrations, LPA induced a $[Ca^{2+}]_c$ signal with an initial peak-like component, followed by a long-lasting $[Ca^{2+}]_c$ elevation, which depended partially on extracellular Ca^{2+} (Fig. 3*A*). In contrast to rat microglia, the LPA-induced $[Ca^{2+}]_c$ signals in mouse microglia showed a pronounced desensitization to repeated application of LPA (Fig. 3, *B* and *C*). At concentrations $\leq$1 μM LPA, the number of cells responding to a second or third application was reduced by ~50%; but between LPA applications, the $[Ca^{2+}]_c$ returned to the base line. At 3 μM LPA, the number of cells responding to repeated LPA application was reduced by only ~30%; but after the first LPA application, $[Ca^{2+}]_c$ was maintained at a new steady-state level above the base line, and the transients had a substantially reduced amplitude. Because of this desensitization, the concentration-response graph shown in Fig. 2*B* was obtained from initial responses in separate experiments. In contrast to the rat cells, which showed responses only at concentrations $\geq$30 nM, mouse microglial cells showed responses to LPA concentrations as low as 3 nM.

Source of the $[Ca^{2+}]_c$ Increases in Rat Microglial Cells—LPA-induced increases in $[Ca^{2+}]_c$ have been shown to be due both to influx through the plasma membrane and to inositol 1,4,5-trisphosphate-mediated release from intracellular stores (10,

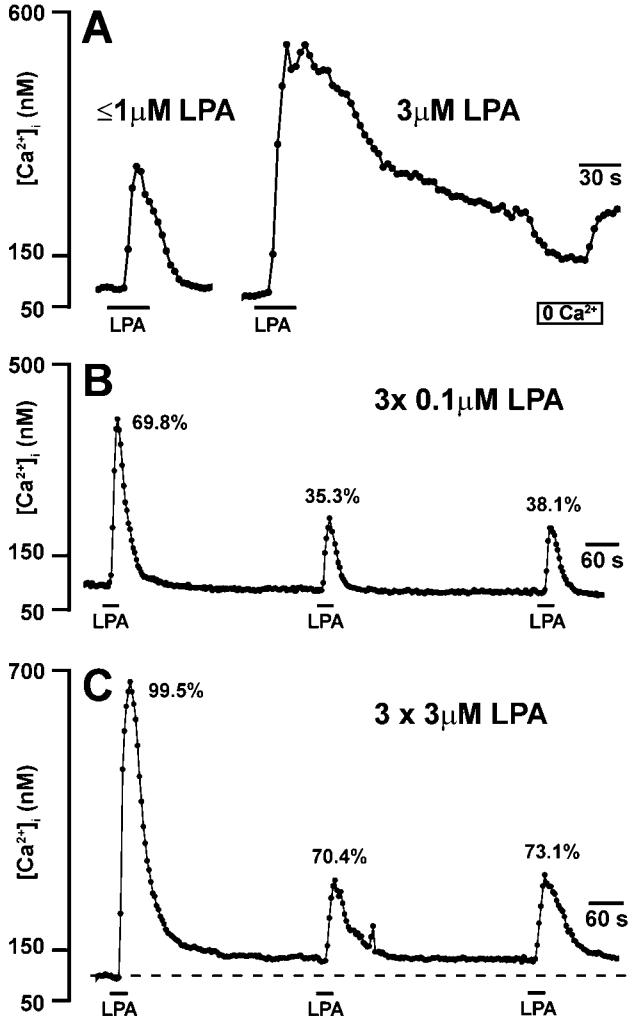

FIG. 3. **LPA-induced $[Ca^{2+}]_c$ signals in cultured mouse microglial cells.** *A*, Fura-2-loaded mouse microglial cells were perfused with varying concentrations of LPA. LPA concentrations ≤1 μM induced a transient $[Ca^{2+}]_c$ signal, whereas 3 μM LPA induced a long-lasting $[Ca^{2+}]_c$ signal, partially dependent on extracellular Ca^{2+}. *B* and *C*, repetitive application of LPA led to reduced signals. At low concentrations of LPA (≤1 μM), the signal returned to the base line between applications (*B*), whereas the first application of 3 μM LPA led to a new elevated $[Ca^{2+}]_c$ steady state, from which cells responded to LPA (*C*). Percentages given correspond to the percentage of cells responding in the experiment shown.

17, 20). To determine the origin of the observed LPA-induced $[Ca^{2+}]_c$ signals in rat microglial cells, 3 μM LPA was applied in Ca^{2+}-free bathing solution (Fig. 4*A*). This treatment completely abolished the $[Ca^{2+}]_c$ transients, suggesting that the LPA-induced $[Ca^{2+}]_c$ signals were entirely of extracellular origin. This hypothesis was further supported by three experiments. First, we observed a normal LPA-induced $[Ca^{2+}]_c$ increase after pretreatment of cells with thapsigargin (500 nM, 30 min) (Fig. 4*B*), a compound that inhibits the endoplasmic Ca^{2+}-ATPase and results in a depletion of endoplasmic Ca^{2+} stores (32). The depletion of the endoplasmic Ca^{2+} stores was confirmed by a lack of response to UTP (100 μM), a metabotropic agonist for microglial cells that stimulates increased $[Ca^{2+}]_c$ through endoplasmic release (31). Second, the LPA-induced $[Ca^{2+}]_c$ transients were blocked by La^{3+} (100 μM), a nonselective cation channel blocker (Fig. 4*C*). Viability of the cells during La^{3+} treatment was demonstrated by the ability of the cells to generate an ATP-induced $[Ca^{2+}]_c$ signal. Third, lidocaine, a sodium channel blocker known to interfere with LPA-induced ion currents (33), reduced the response magnitude and percentage of cells responding to LPA from 95.8 ± 3.8 to 14.8 ± 4.1% (n = 168) (Fig. 4*D*). Removal of lidocaine from the bathing solution restored the LPA-induced $[Ca^{2+}]_c$ responses to 88.7 ± 5.2%. This result further supports the hypothesis that a plasma membrane ion channel is involved in the rat $[Ca^{2+}]_c$ response.

Source of the $[Ca^{2+}]_c$ Increases in Mouse Microglial Cells—In contrast to rat microglial cells, the LPA-induced $[Ca^{2+}]_c$ signal in mouse microglial cells persisted in Ca^{2+}-free buffer, indicating an intracellular release component (Fig. 5*A*). Preincubation with thapsigargin (500 nM, 30 min) blocked the signals at LPA concentrations ≤1 μM, whereas 3 μM LPA induced a $[Ca^{2+}]_c$ transient, which was dependent on the presence of extracellular Ca^{2+} (Fig. 5*B*). This indicates that, in mouse microglial cells, lower concentrations of LPA result in Ca^{2+} release from stores, whereas at high LPA concentrations, an additional plasma membrane influx pathway is recruited. This transmembrane influx was blocked by Ca^{2+}-free buffer, lidocaine (40 mM) (data not shown), or La^{3+} (10 μM) (Fig. 5*C*).

G-proteins Mediating the $[Ca^{2+}]_c$ Signal—To investigate which G-proteins mediate the $[Ca^{2+}]_c$ signal in microglia, cells were preincubated with 1 μg/ml PTX for 2 h. This treatment, which ADP-ribosylates and inhibits G_i/G_o proteins, was shown to be effective in cultured microglial cells (26). PTX did not prevent LPA-induced $[Ca^{2+}]_c$ transients in rat microglial cells (Fig. 4*E*), but completely blocked the response in mouse microglial cells (Fig. 5*D*)

LPA-induced Increases in Microglial Metabolic Activity/Proliferation—Increased metabolic activity and/or proliferation is a common feature of activated microglial cells. To investigate if LPA induces increases in microglial metabolic activity/proliferation, cells were incubated with LPA (1, 3, 10, or 30 μM) for 1 h. Metabolic activity/proliferation was assessed 24 and 48 h later using the WST-1 assay, which relies on the measurement of mitochondrial activity as a readout for metabolic activity and/or proliferation. We found that LPA induced concentration-dependent increases in metabolic activity in mouse microglial cells after 24 and 48 h, whereas rat microglial cells did not show such increases (Fig. 6).

DISCUSSION

Molecular Receptor Characterization—The effects of LPA are mediated through at least three known G-protein-coupled receptors encoded by different genes (lp_{A1}/*Edg2*, lp_{A2}/*Edg4*, and lp_{A3}/*Edg7*) (for review, see Refs. 5 and 6). Although we showed that LPA receptor genes are expressed by cultured microglial cells from mouse and rat, it was surprising to find that different receptor subtypes were expressed by the different species. Primary rat microglial cultures predominantly expressed lp_{A3}, whereas primary mouse microglial cultures predominantly expressed lp_{A1}. The different receptor genes expressed by the different species suggested that different LPA responses might be observed in the different cultures since the receptor subtypes differ somewhat in their coupling specificities (7, 11, 12).

It has previously been shown by Northern blotting that lp_{A1} is expressed in the developing and adult mouse central nervous system (18) and in the adult human brain (34). Although lp_{A1} was shown to co-localize with proteolipid protein in adult brain (indicating expression in oligodendrocytes), double staining was not performed with microglial specific markers (18). Generalized adult brain expression of both lp_{A2} and lp_{A3} is low in both murine and human species (34–36). Nevertheless, the relatively small percentage of microglial cells in regard to the total brain volume (37) may lead to a very low signal on a total brain RNA blot. Thus, it remains possible that these genes are expressed by microglia in adult brain, but Northern blotting is not sensitive enough to pick up the signal. Additionally, the lp_A genes might be up-regulated due to injury-type signals caused

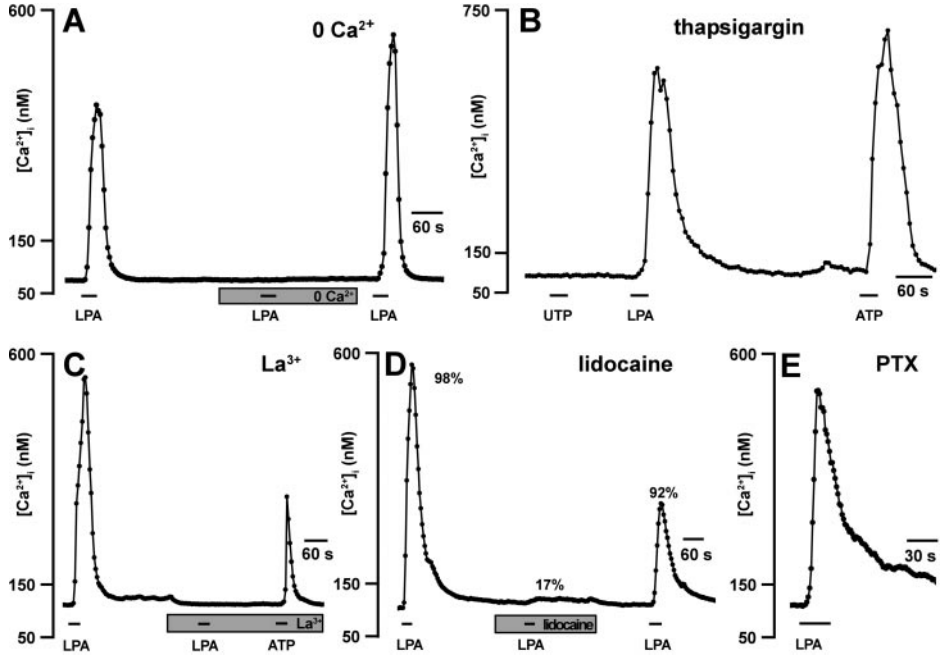

FIG. 4. **Pharmacological modulation of the LPA-induced $[Ca^{2+}]_c$ signal in rat microglial cells.** *A*, application of LPA (3 μM) in Ca^{2+}-free buffer abolished the $[Ca^{2+}]_c$ signal ($n = 135$). *B*, preincubation with thapsigargin (500 nM, 30 min) eliminated metabotropic responses (UTP), but did not abolish the LPA (3 μM)-induced $[Ca^{2+}]_c$ signal, suggesting the involvement of transmembrane Ca^{2+} influx ($n = 157$). ATP (100 μM) served as a viability control. *C*, LPA (3 μM) application also failed to elicit a $[Ca^{2+}]_c$ signal in the presence of La^{3+} (100 μM). Application of ATP (100 μM) served as a control for the viability of the cells ($n = 120$). *D*, lidocaine (40 mM), a sodium channel blocker, reversibly reduced the percentage of responding cells (17% *versus* 98/92%, $n = 80$). *E*, preincubation with PTX (1 μg/ml, 2 h) did not prevent the LPA-induced $[Ca^{2+}]_c$ signal ($n = 92$).

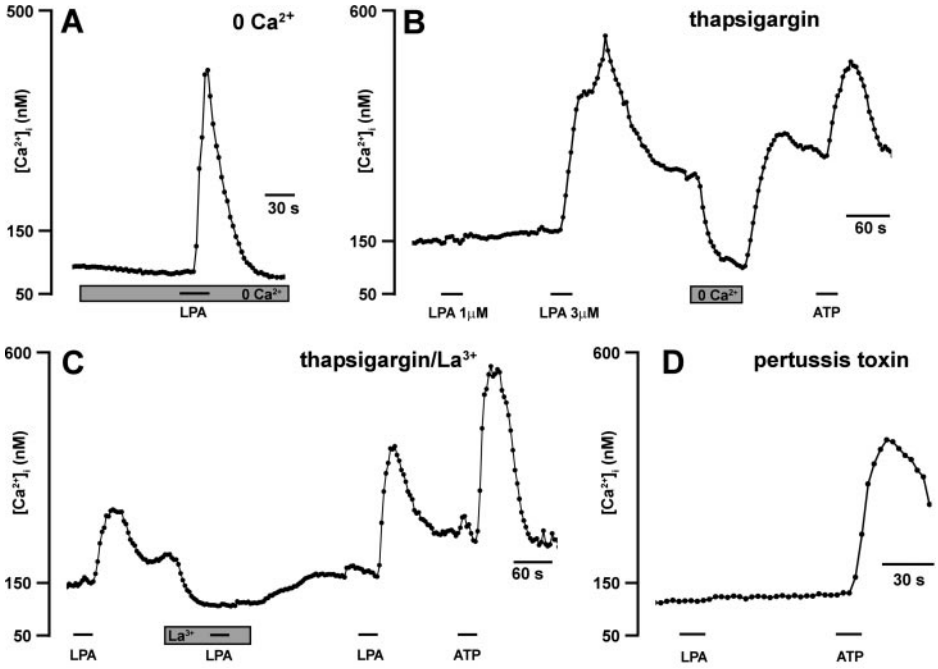

FIG. 5. **Pharmacological modulation of the LPA-induced $[Ca^{2+}]_c$ signal in mouse microglial cells.** *A*, the LPA-induced $[Ca^{2+}]_c$ signal persisted in Ca^{2+}-free medium (3 μM) ($n = 172$). *B*, preincubation with thapsigargin (500 nM, 30 min) blocked LPA-induced responses at 1 μM LPA, but 3 μM LPA induced a $[Ca^{2+}]_c$ signal, which was dependent on external Ca^{2+}, suggesting the involvement of transmembrane Ca^{2+} influx ($n = 65$). *C*, LPA (3 μM) application also failed to elicit a $[Ca^{2+}]_c$ signal in the presence of La^{3+} (10 μM) ($n = 46$). *D*, preincubation with PTX (1 μg/ml, 2 h) blocked the LPA-induced Ca^{2+} signal ($n = 100$). The subsequent application of ATP (100 μM) served as a control for the viability of cells.

by the culturing process (*e.g.* cell dissociation), indicating the expression in microglial cells of a higher activation state.

In the examined microglial cell lines, we found that mouse N9 and BV2 cells had receptor profiles different from those of the primary cells. This might be attributed to cellular changes due to immortalization and suggests caution in extrapolating observations from cell lines to primary cells.

It seems important to keep in mind that the main body of data available on LPA receptor expression (including this study) shows expression at the mRNA level. As the amount of actual protein expressed does not necessarily match the amount of mRNA, one has to be cautious with the interpretation of the data. Nevertheless, the observed differences in cellular responses might indicate an actual difference in functional LPA receptor expression. Further scrutinizing of LPA receptor expression awaits the availability of cross-species receptor antibodies.

To our knowledge, this is the first report showing species-specific expression of LPA receptor genes in a given cell type. However, LPA responses have previously been shown to differ between different species. For example, LPA induces platelet aggregation in humans, but not in rats, and leads to opposite effects on blood pressure upon intravenous injection (38, 39). As the three cloned LPA receptors differ somewhat in their signal transduction properties (7, 12), we tested whether LPA stimulation of microglial cells of different origin (and therefore different LPA receptor expression) led to different responses.

LPA-induced $[Ca^{2+}]_c$ Signals—LPA induced $[Ca^{2+}]_c$ signals in primary cultured mouse as well as rat microglial cells. In rat microglia, LPA induced $[Ca^{2+}]_c$ signals at concentrations as low as 30 nM, eliciting maximal population responses at concentrations of 3 μM. The concentration of LPA required to elicit maximal responses in single cell varied, however. This discrepancy was most likely due to inconsistent solubility of LPA in

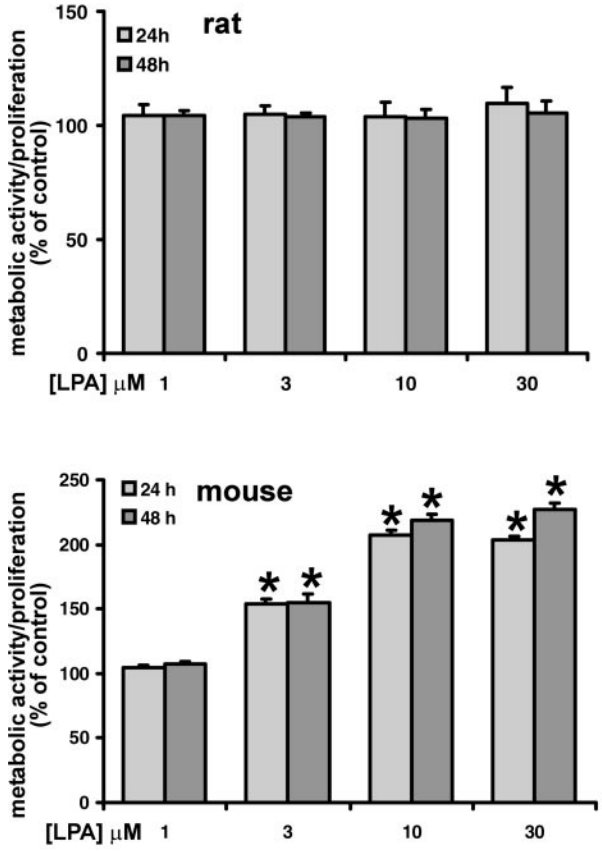

FIG. 6. **LPA-induced changes in metabolic activity/proliferation in rat and mouse microglial cells.** Application of LPA (1, 3, 10, and 30 μM) did not induce any significant change in metabolic activity/proliferation in rat microglial cells. In contrast, the application of LPA to mouse microglial cells led to significant (*, $p < 0.05$) increases in metabolic activity/proliferation at concentrations ≥ 3 μM measured at 24 and 48 h. Metabolic activity/proliferation was assessed using the WST-1 reagent and was concentration-dependent for mouse microglial cells. Data are presented as means ± S.E. ($n \geq 6$).

aqueous solution (40, 41). Nevertheless, in a given population of microglia, recruitment of responding cells was concentration-dependent, and no significant reduction in responses was seen, even at the highest concentrations tested (3 μM). The LPA-induced increase in rat microglial $[Ca^{2+}]_c$ appeared to be due to Ca^{2+} influx via the plasma membrane, as it was abolished in Ca^{2+}-free buffer and did not involve a detectable release from internal stores as shown by the perseverance of the $[Ca^{2+}]_c$ signal despite depletion of internal stores by thapsigargin. Further support of this was provided by the sensitivity of the $[Ca^{2+}]_c$ signals to La^{3+} and lidocaine, two broad-spectrum ion channel blockers. An LPA-gated nonselective cation channel has been described recently in cortical neuroblasts (20), and a similar mechanism might operate in rat microglial cells and lead to the observed $[Ca^{2+}]_c$ influx.

In contrast, the LPA-induced signal in primary mouse microglia persisted in Ca^{2+}-free buffer, but was blocked by thapsigargin, suggesting that it was due to a release from internal stores. Interestingly, this thapsigargin block became incomplete at 3 μM LPA. At this concentration, a $[Ca^{2+}]_c$ signal reappeared, which depended on external Ca^{2+} and was blocked by La^{3+} and lidocaine. This indicated two mechanisms for LPA-induced $[Ca^{2+}]_c$ in mouse microglial cells. At concentrations <3 μM, LPA induced Ca^{2+} release from internal stores, whereas higher concentrations seemed to recruit an additional transmembrane influx pathway. This might reflect activation of LPA receptors other then LP_{A1} (the primary one expressed) or other LP_{A1} receptors with higher binding constants (perhaps due to coupling with distinct signal transduction components).

Other differences in the LPA-induced Ca^{2+} signals between mouse and rat cells were the higher sensitivity of mouse cells, desensitization (which was found only in mouse microglial cells), and the sensitivity to PTX (which also was seen only in mouse cells). This sensitivity to PTX is in accordance with data on heterologous expression of the receptors showing that lp_{A1}, the predominant receptor in mouse microglia, is PTX-sensitive, whereas lp_{A3}, the main receptor in rat microglial cells, is PTX-insensitive (10, 11).

LPA-induced Increase in Microglial Metabolic Activity/Proliferation—Increased metabolic activity/proliferation after LPA stimulation is a common feature in a wide variety of cells. Believed to account for much of the proliferative activity of serum, LPA induces proliferation in hepatocytes (42), B lymphoblasts (43), astrocytes (13), and endothelial cells (44). Interestingly, mouse microglial cells showed a concentration-dependent LPA-induced increase in microglial metabolic activity/proliferation, whereas rat microglia did not change their metabolic activity after LPA stimulation. As rat microglial cells also showed a lower LPA sensitivity in the Ca^{2+} imaging experiments, it might be that the concentrations used were not high enough to stimulate increases in metabolic activity/proliferation in rat cells. Unfortunately, higher concentrations were prevented by the limited solubility of LPA.

Interestingly, the LPA-induced increases in metabolic activity/proliferation coincided with the recruitment of the extracellular calcium influx pathway in mouse microglial cells. Nevertheless, whether this or any other signal transduction pathway is involved in the increase in microglial metabolic activity/proliferation needs to be determined in future experiments. It seems likely that the observed species-specific differences in metabolic behavior were due to the different receptors expressed. Nevertheless, the receptor subtypes that mediate changes in metabolic activity/proliferation have not been determined yet. Our results suggest that, in microglial cells, lp_{A1} mediates changes in metabolic activity/proliferation increases, whereas lp_{A3} does not.

Physiological Implications of Microglial LPA Receptors—LPA-induced morphological changes such as neurite/process retraction and proliferation have been implicated in development and in post-traumatic brain injury (6, 45). It has been postulated that LPA might be a key factor in mediating reactive gliosis and glial scar formation (6). The functional expression of LPA receptors not only in astrocytes, but also in microglial cells, indicates that microglia might be intimately involved in such pathophysiological events. Even though there is variability in the responses of different species, all primary microglia expressed LPA receptors, and both mouse and rat microglia responded to LPA stimulation with $[Ca^{2+}]_c$ signals. Future experiments have to determine if other important parameters of microglial activation, like cytokine expression and phagocytosis, are regulated autonomously or converge toward a common profile.

Conceptually, LPA can leak into the central nervous system during impairment of the blood-brain barrier. Blood-brain barrier impairment may occur rapidly (46) in diverse disorders, including stroke, trauma, inflammation, and seizures (47, 48). Indeed, it was shown that LPA-like activity in the cerebrospinal fluid increases after traumatic brain injury (23). LPA leaking into the central nervous system after blood-brain barrier impairment or due to LPA-induced increased tight junction permeability in cerebral endothelium (24) might be a signal leading to microglial activation. In contrast to cytokines, which are known to activate microglia, but need to be produced by cells, LPA would be immediately present at the site of injury

and might therefore serve as an acute messenger molecule signaling central nervous system injury.

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