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Common Signaling Pathways Link Activation of Murine PAR-1, LPA, and S1P Receptors to Proliferation of Astrocytes

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ABSTRACT

Receptors for the serine protease thrombin and for lysophospholipids are coupled to G proteins and control a wide range of cellular functions, including mitogenesis. Activators of these receptors are present in blood, and can enter the brain during central nervous system (CNS) injury. Reactive astrogliosis, a prominent component of CNS injury with potentially harmful consequences, may involve proliferation of astrocytes. In this study, we have examined the expression and activation of protease activated receptors (PARs), lysophosphatidic acid (LPA) receptors, and sphingosine-1-phosphate (S1P) receptors on murine astrocytes. We show that activation of these three receptor classes can lead to astrogliosis in vivo and proliferation of astrocytes in vitro. Cultured murine cortical astrocytes express mRNA for multiple receptor subtypes of PAR (PAR-1–4), LPA (LPA-1–3) and S1P (S1P-1, -3, -4, and -5) receptors. Comparison of the intracellular signaling pathways of glial PAR-1, LPA, and S1P receptors indicates that each receptor class activates multiple downstream signaling pathways, including Gq/11-directed inositol lipid/Ca^{2+} signaling, Gi/o activation of mitogen-activated protein kinases (MAPK) (extracellular signal-regulated kinase 1/2 and stress activated protein kinase/c-jun N-terminal kinase, but not p38), and activation of Rho pathways. Furthermore, activation of these different receptor classes can differentially regulate two transcription factor pathways, serum response element and nuclear factor of activated T cells. Blockade of Gi/o signaling with pertussis toxin, MAPK activation with 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene (U0126), or Rho kinase signaling with *R*-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexane carboxamide (Y27632) can markedly reduce the proliferative response of glial cells to PAR-1, LPA, or S1P receptor activation, suggesting that each of these pathways is important in coupling of receptor activation to glial proliferation.

Cerebrovascular insults such as traumatic brain injury and stroke cause a breakdown of the blood-brain barrier and thus allow blood constituents direct access to neuronal and glial cells. These insults often result in cell death at and around the site of injury, followed by reactive astrogliosis or glial scarring. Although the functional role of glial scarring is not entirely clear, it is postulated to serve as a mechanism to isolate the damaged region. In addition, the formation of a glial scar and subsequent expression of growth-inhibitory proteoglycans may interfere with neuronal repair or axonal regeneration in the CNS (Davies et al., 1996, 1999; McKeon et al., 1999). Therefore, understanding the factor(s) that initiate and control glial scarring and astrocyte proliferation may lead to therapeutic advances in the treatment of CNS injury. Because activators of protease receptors and lysophospholipid receptors are present in blood, we have examined the biochemical mechanisms that could potentially link activation of these receptors during blood-brain barrier breakdown to astrocyte proliferation.

Although long known for its role in blood coagulation, thrombin plays an important role in CNS injury (Gingrich and Traynelis, 2000; Xi et al., 2003). The thrombin precursor prothrombin, its activating enzyme Factor Xa, as well as thrombin inhibitors and thrombin receptors are all present in the CNS (Deschepper et al., 1991; Dihanich et al., 1991; Vaughan and Cunningham, 1993; Weinstein et al., 1995;

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ABBREVIATIONS: CNS, central nervous system; PAR, protease activated receptor; MAPK, mitogen activated protein kinase; LPA, lysophosphatidic acid; S1P, sphingosine-1-phosphate; LPL, lysophospholipid; BSA, bovine serum albumin; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenyltio)butadiene; Y27632, *R*-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexane carboxamide; U73122, 1-(6-{[17-3-methoxyestra-1,3.5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione; PTX, pertussis toxin; ERK, extracellular signal-regulated kinase; SAPK/JNK, stress activated protein kinase/c-jun N-terminal kinase; PCR, polymerase chain reaction; GFAP, glial fibrillary acidic protein; TBS-T, Tris-buffered saline-Tween 20; NFAT, nuclear factor of activated T cells; SRE, serum response element; PLC, phospholipase C; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; GPCR, G protein-coupled receptor; RT, reverse transcriptase.

Shikamoto and Morita, 1999). Direct infusion of thrombin into rat caudate nucleus causes an increase in reactive gliosis that resembles scar formation (Nishino et al., 1993). The cellular actions of thrombin can often be attributed to the presence of protease-activated receptors (PARs). Of the four known PAR family members, thrombin can activate PAR-1, PAR-3, and PAR-4. Studies on cultured astrocytes demonstrate that thrombin is mitogenic (Grabham and Cunningham, 1995) and suggest that proliferation is probably mediated via activation of PAR-1 but not PAR-3 or PAR-4 (Wang et al., 2002a,b). PAR-1 is a pleiotropic G protein-coupled receptor capable of activating members of the Gi, Gq, and G12/13 families of G proteins (Offermanns et al., 1994; Ogino et al., 1996; Mao et al., 1998). Therefore, a large variety of cellular responses including Gq/11-mediated phosphoinositide turnover and Ca^{2+} signaling, Gi/o-mediated activation of mitogen-activated protein kinase (MAPK) cascades and G12-mediated activation of Rho-serum response element (SRE) pathways have been attributed to PAR-1 (see Macfarlane et al., 2001, for review). Although previous studies indicate that recruitment of the MAPK, phospholipase Cβ, and Gi/o pathways seem to be involved in the coupling of PAR-1 to astrocyte proliferation (Wang et al., 2002a), a full understanding of the signal transduction mechanisms used by PAR-1 to mediate proliferation remains to be elucidated.

In platelets, thrombin activation results in the production of the bioactive lipids, lysophosphatidic acid (LPA), and sphingosine-1-phosphate (S1P) (Sano et al., 2002). These lipids are transported in blood bound to albumin and are thus normally excluded from the CNS. Many of the biological effects of LPA and S1P are attributable to a family of lysophospholipid (LPL) receptors formerly known as EDG receptors. Three LPL receptors designated LPA-1, -2, and -3 display specificity for LPA and five LPL receptors designated S1P-1 through S1P-5 display specificity for S1P, although high concentrations of LPA can activate the S1P-1 receptor (Lee et al., 1998; Fukushima et al., 2001; Takuwa et al., 2002). Similar to PARs, the LPL receptors are coupled to multiple intermediary G protein pathways. Whereas LPA-1 and LPA-2 couple to Gi, Gq, and G12, LPA-3 seems to couple to Gi/o and Gq/11 but not G12 (Fukushima et al., 2001; Takuwa et al., 2002). Similarly, all of the S1P receptors seem to recruit Gi/o. S1P-2 and S1P-3 additionally activate Gq/11 and G12, whereas the S1P-4 and S1P-5 receptors additionally activate G12 but not Gq/11 (Siehler and Manning, 2002). LPA and S1P have been shown to regulate a wide variety of cellular events including cytoskeletal reorganization, differentiation, chemotaxis, and secretion. However, a hallmark of LPA and S1P action in many cells is the induction of proliferation, and these lipids are thus considered major growth factors of serum.

During short-term injury, the CNS is exposed not only to thrombin but also to the products of thrombin's action on platelets, LPA, and S1P. In addition, increasing evidence supports a role for LPA and S1P as paracrine or autocrine mediators released from multiple cell types (Johnson et al., 2002; Xie et al., 2002a,b). LPA is abundant in rat brain (Das and Hajra, 1989), and physiologically active concentrations of LPA have been detected in cerebrospinal fluid in an experimental model of hemorrhagic injury (Tigyi et al., 1995). LPA and S1P have been shown to induce proliferation of astrocytes in culture (Keller et al., 1997; Ramakers and Moolenaar, 1998; Tabuchi et al., 2000; Pebay et al., 2001; Steiner et al., 2002; Yamagata et al., 2003), although some reports indicate that LPA is not a mitogen for astrocytes (Tigyi et al., 1994; Fuentes et al., 1999; Pebay et al., 1999).

In this study, we examine in parallel the capacity of these three classes of receptor (PAR-1, LPA, S1P) to induce mitogenesis in cultured astrocytes and reactive astrogliosis in vivo. We further compare in parallel their coupling to intracellular signaling pathways. Our results suggest that PAR-1, LPA, and S1P each recruit multiple parallel G protein pathways and implicate roles for a Gi/o-MAPK pathway and Rho-SRE pathway in coupling each of these agents to astrocyte proliferation.

Materials and Methods

Materials. 1-Oleoyl-2-hydroxy-*SN*-glycero-3-phosphate (lysophosphatidic acid, LPA) and S1P were purchased from Avanti Polar Lipids (Alabaster, AL). Lipid-free bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). TFLLR-NH_2 (hereafter TFLLR) was synthesized at the Emory University Microchemical Facility. Tissue Tec was purchased from VWR (West Chester, PA). Vectashield and goat serum were purchased from Vector Laboratories (Burlingame, CA). [^{3}H]Thymidine was purchased from Amersham Biosciences (Piscataway, NJ). [*myo*-^{3}H]Inositol was purchased from PerkinElmer Life Sciences (Boston, MA). Fura-2 acetoxymethyl ester was purchased from Molecular Probes, Inc. (Eugene, OR) and U0126 was obtained from Promega (Madison, WI). Thrombin, Y27632, 2-amino ethoxydiphenyl borate, bisindolylmaleimide, and U73122 were purchased from Calbiochem (San Diego, CA). Pertussis toxin (PTX) was purchased from List Biologicals (Campbell, CA). p44/42 MAPK [extracellular signal regulated kinase (ERK) 1/2] antibody, phospho-p44/42 MAPK (Thr202/Tyr204) antibody, phospho-SAPK/JNK (Thr183/Tyr185) antibody, and phospho-p38 MAPK (Thr180/Tyr182) antibody were purchased from Cell Signaling Technology (Beverly, MA). Heat-inactivated horse serum, fetal bovine serum, TRIzol reagent, SuperScript II RNase H^- reverse transcriptase, oligo(dT), dNTP, and RNase OUT were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-glial fibrillary acidic protein (GFAP) was obtained from Accurate Chemical and Scientific Corporation (Westbury, NY). Texas Red goat anti-rabbit IgG was purchased from Molecular Probes. Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio-Rad (Hercules, CA).

Preparation of LPA and S1P. Lipids were prepared fresh in lipid-free 0.1% BSA on the day of the experiment. For S1P, the dry powder was suspended in chloroform and then evaporated under nitrogen. The appropriate volume of water (containing 0.1% lipid-free BSA to stabilize the lipids) was added. Lipids were resuspended by sonication and further diluted to 100× stocks in 0.1% lipid-free BSA. Addition of lipid-free BSA (0.1%) at a 1:100 dilution had no effect compared with water alone for all of the assays tested.

In Vivo Injection of Agonist and GFAP Immunostaining. All procedures involving the use of animals were reviewed and approved by the Emory University IACUC. C57BL/6 male mice (90 days old) from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) were anesthetized with 8% chloral hydrate administered i.p. at 0.4 g/kg. Animals were placed on a homeothermic blanket, and rectal temperature was maintained between 36.2 and 37°C. The head was immobilized in a small animal stereotaxic device (SAS 75; Cartesian Research Inc., Sandy, OR), a scalpel was used to expose the skull, and a hole was drilled with a 0.0087-inch carbide bit at 1.000 mm rostrally, 2.150 mm laterally, and 0.500 mm ventrally to bregma. A 33-gauge needle was inserted vertically 3.450 mm into a predrilled hole and allowed to reside in place for 2 min. Injections of 0.1% lipid-free BSA in phosphate-buffered saline (PBS) at pH 7.4 containing 1 nmol of S1P, 10 nmol of LPA, or 1 pmol of thrombin were made

at 0.1 μl/min for 5 min (total volume, 0.5 μl). After injection, the needle was left in place for 5 min, removed, and the skin was sutured. Animals were allowed to survive 5 days and were then injected with sodium pentobarbital (390 mg/ml) and transcardially perfused with ice-cold 4% paraformaldehyde in PBS for 10 min. The brain was removed and placed in 4% paraformaldehyde for 24 h and cryoprotected in 20% sucrose at 4°C for 24 h. The brain was embedded with Tissue Tec, and stored at −50°C. Embedded tissue was warmed to −20°C, and 10-μm sections were cut on a cryostat (CM3050; Leica, Wetzlar, Germany) and placed on gelatin-coated glass slides. Slices were incubated in 4% paraformaldehyde for 10 min and washed three times in PBS. Sections were incubated in blocking solution composed of 3% goat serum, 0.5% BSA, and 0.25% Triton X-100 in PBS for 1 h at 23°C. Sections were incubated with 1:100 rabbit anti-GFAP in blocking buffer overnight at 4°C. Sections were subsequently washed three times in PBS and incubated with streptavidin-conjugated Texas Red secondary antibody in blocking buffer for 1 h at 23°C. Sections were washed three times, coverslip-coated using Vectashield fluorescent mounting media, and stored in the dark at −20°C until fluorescent images were acquired with a Hamamatsu C4742−95 camera using Openlab software (Improvision).

Primary Culture of Astrocytes. Cultured astrocytes were prepared from P1-P3 postnatal mice. The cerebral cortex was dissected free of adherent meninges, and the tissue was minced and dissociated into single-cell suspension by trituration through a Pasteur pipette. Dissociated cells were plated into multiwell plastic plates coated with 100 μg/ml poly-D-lysine using Dulbecco's modified Eagle medium supplemented with 25 mM glucose, 10% heat inactivated horse serum, 10% heat inactivated (55°C for 30 min) fetal bovine serum, 2 mM glutamine, and a 1:100 dilution of penicillin-streptomycin. After 2 to 4 days in culture, the cells were vigorously shaken to remove all loosely adherent cells. Cultures were maintained at 37°C in a humidified 5% CO_2-containing atmosphere until they reached 50% confluence. Homogenous populations of astrocytes were verified by immunofluorescence staining with GFAP (data not shown).

[^{3}H]Thymidine Incorporation and Cell Proliferation. Primary cultures of astrocytes grown in 24-well plates were serum-starved for 18 to 24 h in the absence or presence of pharmacological inhibitors as noted. Cells were then challenged with agonist for a 24-h period. During the final 2 h of agonist challenge, [^{3}H]thymidine was added to a final concentration of 1 μCi/ml. Cells were washed in ice-cold PBS before the addition of 20% trichloroacetic acid for 30 min at 4°C. Cells were again washed in PBS, and the acid-insoluble material was lysed in 0.1 N NaOH/1% SDS. [^{3}H]Thymidine in lysates was measured by scintillation counting. For measurement of cell proliferation, primary cultures of astrocytes were seeded at equal density in 24-well plates and challenged with agonist for 24 h. Astrocytes were resuspended by treatment with trypsin and counted directly by visual inspection using a hemacytometer.

RT-PCR for Receptor mRNA. Total RNA was extracted from primary astrocyte cultures in 100-mm culture dishes with TRIzol reagent per the manufacturer's directions. Poly A^+ mRNA was purified from total RNA using Oligotex mRNA Kits (QIAGEN, Valencia, CA) and suspended in a 60-μl volume. Reverse transcription was performed on 10-μl aliquots of astrocyte mRNA in the presence or absence of SuperScript II RNase H^- reverse transcriptase per the manufacturer's directions using oligo(dT), dNTP, and RNase OUT from Invitrogen. Aliquots of cDNA samples (2 μl) were used for PCR using PCR SuperMix. Sense and antisense primers (1 μM) designed for murine PAR and LPL receptors are shown in Table 1. Samples were cycled by an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 0.5 min, annealing at 55°C for 0.5 min, and elongation at 72°C for 1 min, and a primer extension at 72°C for 5 min. PCR fragments were separated by agarose gel electrophoresis and identified by ethidium bromide staining.

[^{3}H]Inositol Phosphate Production. Primary astrocytes were serum-starved for 18 to 24 h in complete Dulbecco's modified Eagle medium containing 4 μCi/ml [^{3}H]inositol in the absence or presence of pharmacological inhibitors as described in the text. Cells were placed in media supplemented with 5 mM LiCl, challenged with agonist, and [^{3}H]inositol phosphates were isolated by ion exchange chromatography as described previously (Heximer et al., 1999).

Calcium Mobilization in Intact Astrocytes. Cultured astrocytes were incubated with 5 μM Fura2 acetoxymethyl ester for 30 min at room temperature. Cells were then placed on a microscope stage for imaging of intracellular calcium concentration. External solution contained 150 mM NaCl, 10 mM HEPES, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5.5 mM glucose, pH 7.3, and 325 mOsm. Intensity images of 510 nm wavelength were taken at 340- and 380-nm excitation wavelengths, and the two resulting images were taken

TABLE 1
PCR primers
SIP-2 primers were as outlined in Pebay et al. (2001).

Receptor	Accession Number	Strand	Primer
PAR-1	L03529	S	5′-gccccggcgcttgctgat-3′
		AS	5′-caccacggccggcttcttga-3′
PAR-2	Z48043	S	5′-ggcgtggctgctgggaggtatc-3′
		AS	5′-cggcggggtgtttcttcttcgtt-3′
PAR-3	U92972	S	5′-ccacggctcacccttttcacatac-3′
		AS	5′-tccccaggcacagagcaataaga-3′
PAR-4	AF080215	S	5′-atgatgcgctgcccctgactga-3′
		AS	5′-cctccggctggcgacacaacata-3′
LPA-1	AH006409	S	5′-aaccgccgcttccatttccct-3′
		AS	5′-cagatgcagttccagcccac-3′
LPA-2	AF218844	S	5′-ccgtgtggtcacactcatcgt-3′
		AS	5′-tgcggcgcatctcagcatctcg-3′
LPA-3	AF293845	S	5′-gaattgcctctgcaacatctc-3′
		AS	5′-gagtagatgatggggttca-3′
S1P-1	MMU40811	S	5′-accacaagcactatattct-3′
		AS	5′-ctaacagtagtaggatgaag-3′
S1P-3	AB028143	S	5′-atatgatgccaacaagaagc-3′
		AS	5′-gaggaagaggataaaaagtg-3′
S1P-4	MMU6074	S	5′-gctatgtgctcttttgtgtggt-3′
		AS	5′-gtagatgagaggattaatggct-3′
S1P-5	AF327535	S	5′-cgctgggctggaactgcttaggac-3′
		AS	5′-gtggcgcagatctcggttggtgaa-3′

S, sense; AS, antisense.

from individual cells for ratio calculations. Axon Imaging Workbench version 2.2.1 (Axon Instruments, Union City, CA) was used for acquisition of intensity images and conversion to ratios.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis. Primary cultures of astrocytes were grown in six-well format and serum-starved in the absence or presence of inhibitors as indicated before the addition of the agonists as described. Intracellular signaling reactions were stopped by the addition of 300 μl of hot (80°C) sample buffer. Aliquots (50 μl) of lysates were boiled in SDS sample buffer, subjected to SDS-polyacrylamide gel (12%) electrophoresis and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked at room temperature for 1 h in Tris-buffered saline containing 5% nonfat milk. Antibody to total ERK 1/2 (1:300), phosphorylation-specific ERK 1/2 antibody (1:1000), phosphorylation-specific SAPK/JNK antibody (1:250), or phosphorylation-specific p38 MAPK antibody (1:250) was diluted in Tris-buffered saline supplemented with 0.1% Tween (TBS-T) and incubated with the membranes overnight at 4°C. Membranes were washed with TBS-T and incubated for 1 h in horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 in TBS-T). Membranes were again washed and immunoreactive proteins detected by enhanced chemiluminescence. Total ERK 1/2 immunoreactivity was equal under each experimental condition within each experiment. For experiments with GFAP antibody, cells were scraped into lysis buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Samples were assayed for protein, and equal aliquots (20 μg) of lysate were boiled in SDS sample buffer before SDS-polyacrylamide gel electrophoresis and transfer to Immobilon-P membranes. Immunoblotting was determined as described above with 1:1000 rabbit anti-GFAP primary antibody.

Transcription Factor Luciferase Reporters. Infectious retroviral supernatants were prepared from Phoenix producer cells transfected with retroviral luciferase reporter plasmids containing binding elements for NFAT and SRE as described previously (Abbott et al., 2000). Green fluorescent protein-containing retroviral plasmids were run in parallel and served as controls. Primary astrocytes in six-well format were infected with three rounds of retrovirus exposure resulting in 30 to 70% infection efficiency as assessed by green fluorescent protein. Two to 3 days after infection, astrocytes were passaged (1 to 4 by surface area) onto poly-D-lysine–coated 24-well plates. Two to 7 days after passage, cells were serum-starved overnight before application of agonist for the time indicated in the text. Reactions were stopped, and luciferase activity was monitored on a luminometer using a luciferase assay system (Promega).

Dose Response Analysis. Dose response curves were analyzed using Prism software (GraphPad, San Diego, CA) and data fit by nonlinear regression to a variable slope sigmoidal dose-response curve.

Results

Thrombin, LPA, and S1P Increase Astrocyte GFAP Immunoreactivity in Vivo. The defining feature of reactive astrocytes involved in glial scar formation is the expression of GFAP. To examine whether activation of PAR or LPL receptors might participate in glial scar formation, we tested whether PAR and LPL agonists injected in vivo can induce GFAP expression. Although the effects of PAR agonists LPA and S1P on astrocytes in vitro have been reported, few data exist describing the effects of these agents in vivo. To compare the effects of thrombin, LPA, and S1P on expression of GFAP-positive astrocytes in vivo, each compound was directly injected into the striatum of 90-day-old mice, and astrocyte reactivity was determined by GFAP immunostaining performed 5 days after injection (see *Materials and Methods*). Injection of thrombin (1 pmol in 0.5 μl), LPA (10 nmol in 0.5 μl), and S1P (1 nmol in 0.5 μl) all increased the number of GFAP-positive cells compared with either injection of buffer alone or with contralateral striatum (Fig. 1). Coinjection of 50 pmol/0.5 μl of the thrombin receptor antagonist BMS200261 (Bernatowicz et al., 1996) with thrombin (1 pmol/0.5 μl) markedly reduced the gliotic response in mice (n = 3, data not shown); BMS200261 alone had no effect on GFAP immunoreactivity (n = 2). Cresyl violet staining in parallel slices was homogenous, thereby confirming that these changes in astrocyte reactivity were not the result of nonspecific injury at the site of injection (data not shown).

Thrombin, TFLLR, LPA, and S1P Stimulate Astrocyte Proliferation in Vitro. A key aspect of astrocyte reactivity is a proliferative response. Therefore, the capacity of thrombin, LPA, and S1P to induce a mitogenic response was evaluated in astrocytes in culture. Previous studies have determined that PAR-1, but not PAR-3 or PAR-4, is primarily responsible for proliferative responses to thrombin in astrocytes; activation of the thrombin-insensitive trypsin receptor PAR-2 initiates a moderate proliferative response (Wang et

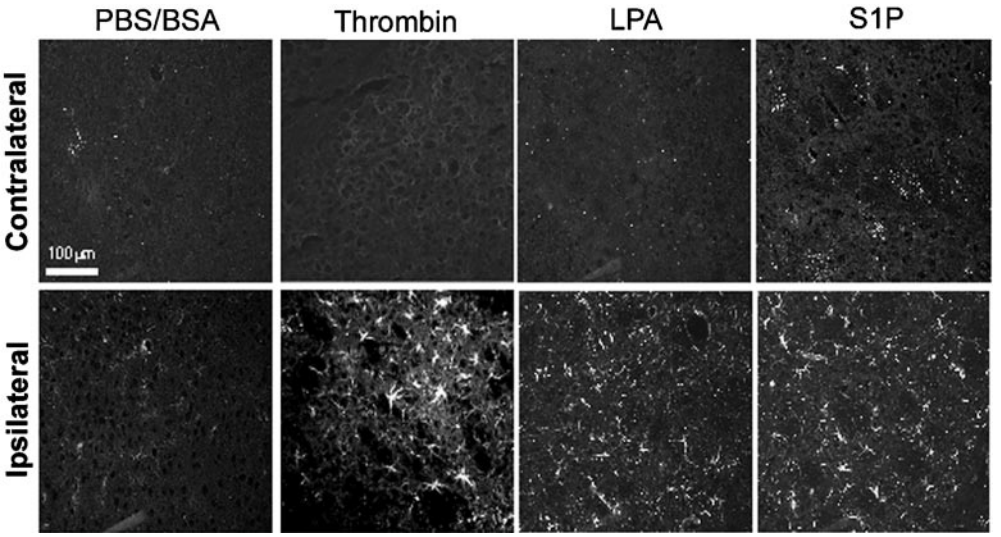

Fig. 1. In vivo injection of thrombin, LPA. or S1P induces astrogliosis. Vehicle (0.5 μl, PBS/BSA 0.1%), thrombin (1 pmol/0.5 μl), LPA (10 nmol/0.5 μl), or S1P (1 nmol/0.5 μl) was injected over 5 min directly into the striata of 90-day-old mice. Mice were sacrificed after 5 days and slices were prepared for immunostaining of GFAP using streptavidin-conjugated Texas Red secondary antibody (see *Materials and Methods*). Images from both a noninjected site (contralateral) and the injected site (ipsilateral) are shown. Data shown are representative of experiments performed in three different animals. White scale bar, 100 μm.

al., 2002a). Therefore, we also compared the effects of the selective PAR-1–activating peptide TFLLR, which mimics the new N terminus revealed by thrombin cleavage at Arg_{41} (Hollenberg et al., 1997). Primary cultures of astrocytes were challenged with either 50 pM thrombin, 30 μM TFLLR, 30 μM LPA, or 10 μM S1P, and proliferation was monitored by the incorporation of [^{3}H]thymidine and direct counting of cells. Thrombin, TFLLR, and LPA induced a 5- to 6-fold increase in [^{3}H]thymidine incorporation, whereas S1P induced only a 3-fold increase (Fig. 2). The increase caused by S1P was measurably smaller than that caused by TFLLR and LPA (statistically significant, $p < 0.05$, Neuman-Keuls Test). The number of astrocytes also increased measurably over 24 h in response to the same concentration of each agonist [thrombin, 2.0 ± 0.4-fold (S.E.M.) compared with basal; TFLLR, 2.0± 0.4-fold; LPA 1.8 ± 0.3-foldl; and S1P, 1.6 ± 0.4-fold]. No changes in normalized GFAP expression (immunoreactive GFAP/total protein) were observed by immunoblot analysis in cultured astrocytes treated with these agents (data not shown). Because thrombin and TFLLR have similar effects on astrocyte proliferation and evidence suggests that thrombin actions are mediated by PAR-1 (Wang et al., 2002a), subsequent signaling studies focused on the comparative effects of TFLLR, LPA, and S1P on astrocyte signaling events.

Multiple PAR, LPA, and S1P Receptor Subtype mRNAs Are Expressed in Astrocytes. Although TFLLR requires PAR-1 to induce a proliferative response and is a specific PAR-1 agonist, the effects of LPA and S1P on proliferation could be mediated by receptor-dependent and/or receptor-independent effects of these agents (Van Brocklyn et al., 1998; Spiegel and Milstien, 2000; Hooks et al., 2001). Therefore, the presence of mRNA for these receptors was analyzed by RT-PCR. mRNA for all four known PAR subtypes and all three known LPA subtypes was detected in the astrocytes (Fig. 3, A and B). Of the five known S1P receptors, mRNA for S1P1, S1P3, S1P4, and S1P5 was detected (Fig. 3C). Although the mRNA for S1P2 was readily detected in NIH3T3 fibroblasts (data not shown), this receptor mRNA was not detected in cortical astrocytes (Fig. 3C).

Cellular Signaling Responses to TFLLR, LPA, and S1P in Astrocytes. The presence of functional LPA and S1P receptors was determined by monitoring the inositol phosphate production that occurs in response to receptor-mediated activation of the Gq/11-phospholipase C (PLC) pathway. Application of either LPA or S1P induced a time-dependent accumulation of inositol phosphates (Fig. 4A) demonstrating the presence of Gq/11-coupled LPA and S1P receptors. Similarly, TFLLR induced activation of the Gq/11-PLC pathway. Although S1P had the lowest relative activity in [^{3}H]thymidine assays, S1P displayed the highest intrinsic activity for inositol phosphate production (Figs. 2 and 4B). TFLLR, LPA, and S1P also stimulated a marked rise in intracellular Ca^{2+} levels in individual astrocytes (Fig. 4C). Stimulated increases in intracellular Ca^{2+} were similar in both magnitude (5-fold over resting basal) and duration for each agonist. Intracellular Ca^{2+} levels peaked within 30 s and returned to baseline levels after 4 min for TFLLR, LPA (Fig. 4C); after S1P treatment, Ca^{2+} levels also peaked within 30 s and declined but remained elevated after 4 min (Fig. 4C).

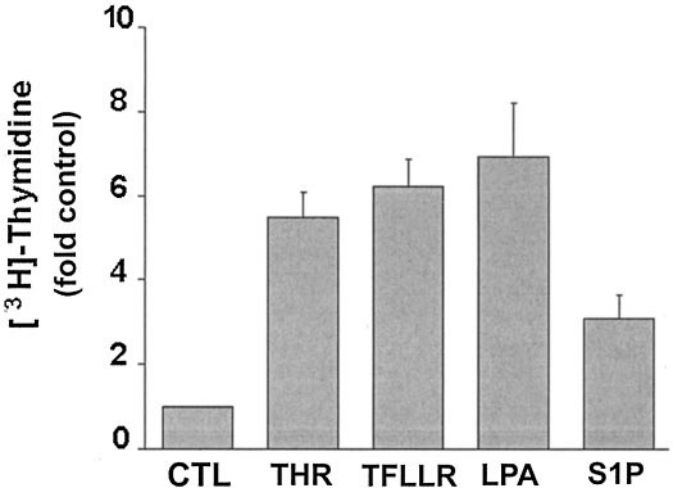

Fig. 2. Thrombin, TFLLR, LPA, and S1P induce proliferation of astrocytes in vitro. Primary cultures of cortical astrocytes were serum-starved for 18 to 24 h before treatment with vehicle (CTL), 50 pM thrombin, 30 μM TFLLR, 30 μM LPA, or 10 μM S1P for an additional 24 h. [^{3}H]Thymidine was added for the final 2 h before recovery of [^{3}H]thymidine in the acid-insoluble material. Data are reported as incorporation of [^{3}H]thymidine normalized to vehicle control (CTL) and are mean ± S.E.M. ($n = 6$). [^{3}H]Thymidine incorporation for control is 1220 ± 90 cpm.

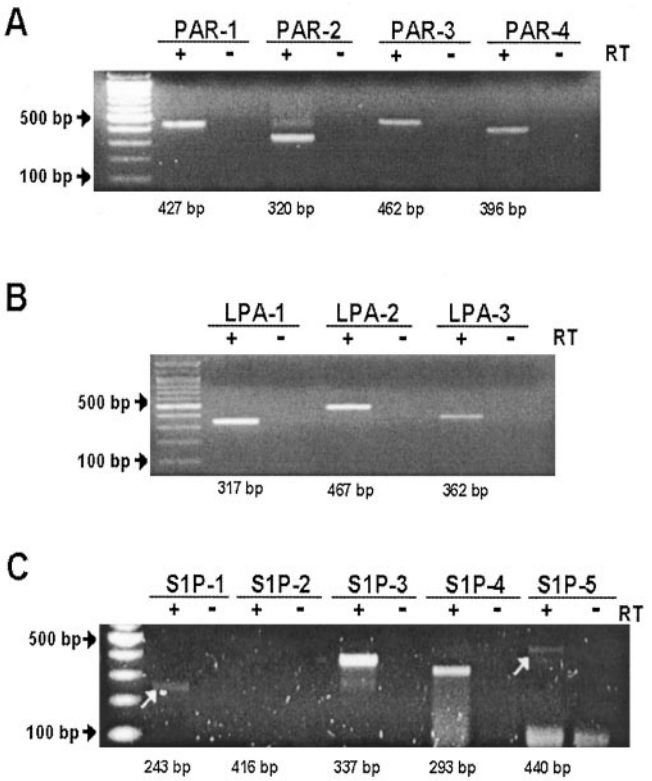

Fig. 3. Identification of PAR, LPA, and S1P receptor subtypes in cortical astrocytes by RT-PCR. mRNA was purified from total RNA isolated from primary astrocyte cultures. RT-PCR was performed to evaluate the presence of mRNA for the four known PARs (PAR1–4) (A), three known LPA receptors (LPA-1, formerly EDG2; LPA-2, formerly EDG4; LPA-3, formerly EDG7) (B), and five known S1P receptors (S1P-1, formerly EDG1; S1P-2, formerly EDG5; S1P-3, formerly EDG3; S1P-4, formerly EDG6; S1P-5, formerly EDG8) (C). PCR was performed in the absence (−) of the reverse transcriptase (RT) to verify that contaminating genomic DNA was not present in the samples. Numbers below the gels indicate predicted size in base pairs of the PCR product for that sample. The standards are in increments of 100 base pairs. Figures shown are representative of two to three independent experiments performed for each receptor.

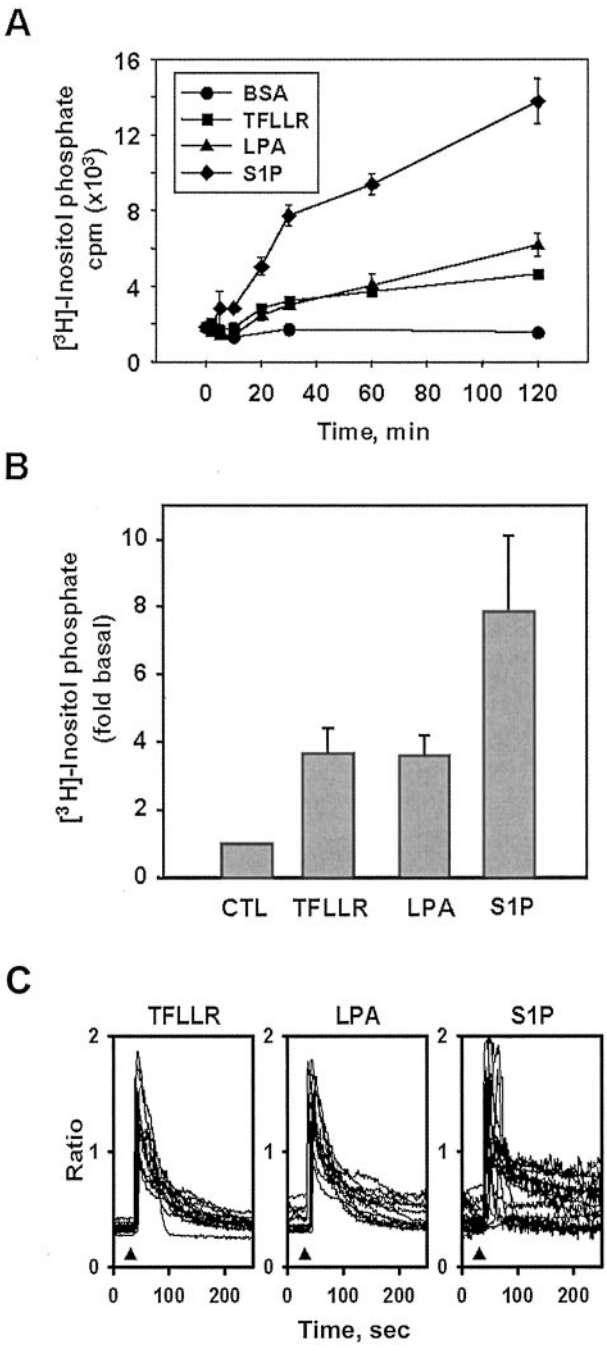

Fig. 4. TFLLR, LPA, and S1P activate the Gq/11-inositol lipid/Ca^{2+} signaling pathway in cortical astrocytes. Cortical astrocytes were serum starved for 24 h in the presence of [^{3}H]inositol. A, cells were treated for indicated times with vehicle (BSA), 100 μM TFLLR, 30 μM LPA, or 10 μM S1P before isolation of [^{3}H]inositol phosphates in the presence of Li^+. Data are reported as cpm recovered per well in triplicate and are presented as mean ± S.E.M. Data are from a single experiment performed in triplicate and representative of three experiments. B, astrocytes were treated with vehicle (CTL), 100 μM TFLLR, 30 μM LPA, or 10 μM S1P for 30 min and [^{3}H]inositol phosphates recovered. Data are normalized to [^{3}H]inositol phosphate incorporation with vehicle alone (CTL) and are mean ± S.E.M. from five experiments. Basal [^{3}H]inositol phosphate incorporation is 965 ± 167 cpm (n = 5). C, cultured astrocytes were loaded with Fura-2 and treated with vehicle (CTL), 100 μM TFLLR, 30 μM LPA, or 10 μM S1P for 20 s. TFLLR, LPA, and S1P each stimulate an increase in intracellular Ca^{2+} concentration in astrocytes. Each trace represents changes in Ca^{2+} concentration of a single astrocyte (normalized to resting Ca^{2+} levels), and traces from multiple cells are overlaid (n = 14 for TFLLR, n = 13 for LPA, n = 17 for S1P). ▲, onset of drug applications. Results are representative of two experiments.

To further probe the interrelationship between inositol phosphate production and [^{3}H]thymidine incorporation in astrocytes, dose response relationships were determined. For TFLLR, the potency to induce the downstream proliferative response is approximately 10-fold greater than potency for activation of the upstream PLC response. This leftward shift in the dose-response curve is predicted by signal amplification. In contrast, LPA and S1P are less potent in activating [^{3}H]thymidine incorporation compared with activation of PLC (Fig. 5).

To further assess the signaling pathways activated by TFLLR and the LPLs, immunoblot analysis was used to determine the ability of these agents to induce phosphorylation of MAPK. Phospho-specific antibodies to ERK 1/2, stress activated protein kinase/c-jun N-terminal kinase (SAPK/JNK), and p38 MAPK were used to determine changes in the phosphorylation state of these MAPK family members. All of the agonists tested induced phosphorylation of ERK1/2 and SAPK/JNK, but no changes in p38 phosphorylation were detected. Although the onset of phosphorylation induced by each of these agents was rapid (2 to 5 min), kinetic differences in the sustained activation of the MAPK pathways were noted. LPA induced a prolonged phosphorylation of both MAPK pathways, whereas the effects of S1P were more transient and returned to baseline values at 1 to 2 h. TFLLR displayed differential effects on the MAPKs such that phosphorylation of ERK1/2 was prolonged and that of SAPK/JNK was transient (Fig. 6).

MAPKs and Rho Kinase couple TFLLR, LPA and S1P Responses to Astrocyte Proliferation. To dissect the pathway(s) responsible for the coupling of TFLLR, LPA, or

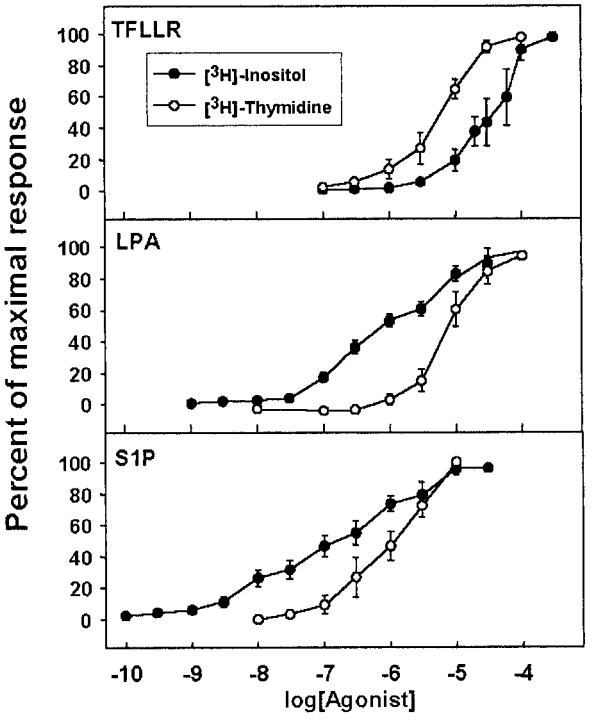

Fig. 5. Dose response for agonist-induced phosphoinositide turnover versus proliferation. Cortical astrocytes were serum-starved for 24 h in the absence (proliferation) or presence (phosphoinositide turnover) of [^{3}H]inositol. Cells were treated with the indicated concentrations of agonists, and proliferation or phosphoinositide turnover was monitored as described for Figs. 2 and 4. Data are mean ± S.E.M., n = 3 to 5.

S1P activation of PAR-1 and LPL receptors to astrocyte proliferation, cultured astrocytes were treated with pharmacological inhibitors of MEK/ERK, Gi/o, PLCβ, inositol phosphate receptor, protein kinase C (PKC), or Rho pathways before agonist challenge. Treatment of cells with 10 μM of the MEK inhibitor U0126 blocked the coupling of each agonist to both ERK1/2 and SAPK/JNK phosphorylation (Fig. 7A). Similarly, pretreatment of cells for 24 h with 0.1 μg/ml PTX to disrupt receptor coupling to Gi partially inhibited the coupling of TFLLR, LPA, and S1P to these MAPK pathways (Fig. 7B). These data suggest that Gi/o proteins contribute to TFLLR- and LPL-mediated activation of MAPK pathways. However, studies using the Rho kinase inhibitor, Y27632, demonstrate that activation of this Rho effector is not required for coupling TFLLR, LPA, or S1P to the phosphorylation of either ERK1/2 or SAPK/JNK (Fig. 7C). In addition, PTX partially inhibits the coupling of TFLLR, LPA, and S1P to accumulation of inositol phosphates, indicating that both Gq/11 and Gi/o mechanisms for phosphoinositide turnover exist in astrocytes. In contrast, Y27632 does not affect the coupling of the agents to phosphoinositide turnover (data not shown).

Although these inhibitors had differential effects on agonist-induced phosphoinositide turnover as well as phosphorylation of MAPK pathways, each disrupted agonist-induced activation of proliferative responses. The MEK inhibitor U0126 completely inhibited TFLLR-, LPA-, or S1P-induced increases in [^{3}H]thymidine incorporation, indicating that the activation of MAPK pathways is necessary for these agonists to activate proliferation in astrocytes. TFLLR-, LPA-, and S1P-induced activation of proliferation was sensitive to both PTX and Y27632. PAR-1 mediated proliferation was inhibited 70% to 80% in the presence of either PTX or Y27632, whereas LPA and S1P responses were inhibited by 40% to 50% in the presence of these inhibitors (Fig. 8).

To further probe the role of Gq-PLC pathways in mediating TFLLR or LPL effects on proliferation, cells were treated with various pharmacological inhibitors of this pathway. Treatment of astrocytes with 10 μM of the PLCβ inhibitor U73112, the minimal concentration we found necessary to block inositol phosphate production, or with 100 μM of the inositol 1,4,5-trisphosphate receptor inhibitor 2-amino ethoxydiphenyl borate, the reported concentration needed to block Ca^{2+} mobilization, was cytotoxic to cultured astrocytes after 6 h and prohibited further proliferative studies with these agents. Treatment of astrocytes with 1 μM concentrations of the PKC inhibitor bisindolylmaleimide, a concentration that completely blocks the activity of many PKC isoforms (Toullec et al., 1991), was not cytotoxic and did not

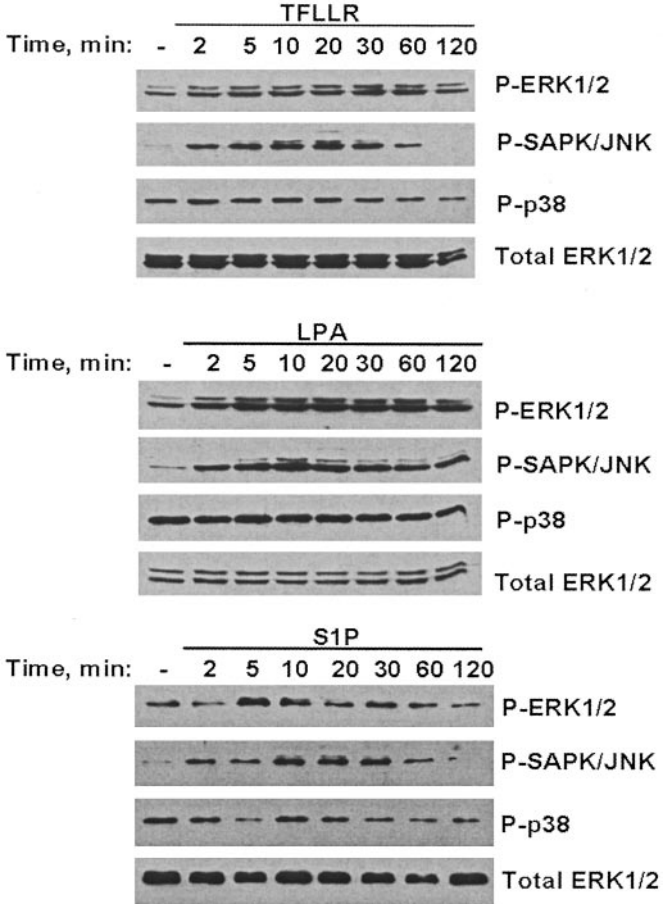

Fig. 6. Time course for TFLLR, LPA, and S1P activation of MAPK pathways. Serum-starved astrocytes were treated with 100 μM TFLLR, 30 μM LPA, or 10 μM S1P for the times shown. Lysates were immunoblotted for phosphorylated proteins (P-ERK1/2, P-SAPK/JNK, P-p38) or total ERK1/2 by immunoblot with the indicated antibody. Equal protein loading of lanes was verified by immunoblot for total ERK1/2. Immunoblots shown are representative of two independent experiments.

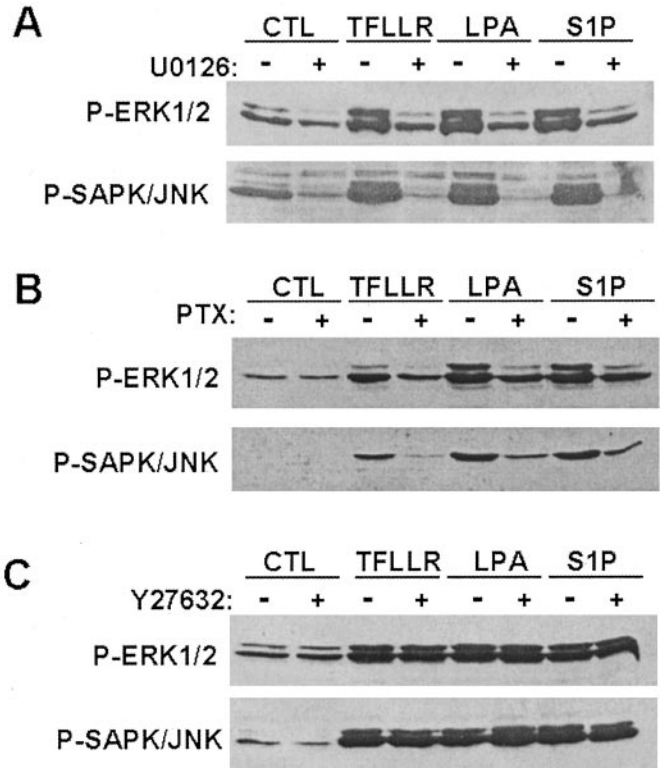

Fig. 7. Effect of signaling inhibitors on TFLLR, LPA, and S1P coupling to MAPK pathways. Astrocytes were serum-starved for 24 h to arrest the growth of cells. A, for analysis of the MEK pathway, cells were treated with or without 10 μM U0126 (a direct MEK inhibitor) for 30 min before addition of agonist. B, for analysis of the Gi pathway, cells were treated with or without PTX (100 ng/ml) for 24 h while serum-starved. C, for analysis of the Rho pathway, cells were treated with or without the Rho kinase inhibitor Y27632 (10 μM) for 24 h while serum-starved. Cells were then treated with vehicle alone (CTL), 100 μM TFLLR, 30 μM LPA, or 10 μM S1P for 5 min, lysed, and immunoblotted with the indicated antibody. Immunoblots shown are representative of two or three independent experiments performed.

alter the capacity of TFLLR, LPA, or S1P to stimulate astrocyte proliferation (data not shown).

TFLLR, LPA, and S1P Activate Two Transcriptional Pathways. Transcription factors serve as downstream effectors of many signal transduction pathways. Activation of the PLC/Ca^{2+} pathway and subsequent activation of calcineurin can initiate transcription through NFAT (Macian et al., 2001). Although each of the agonists studied couples to inositol phosphate production and Ca^{2+} mobilization (Fig. 4), only LPA and S1P can couple to the prolonged activation of NFAT pathways as measured by an NFAT-luciferase reporter, whereas TFLLR does not appreciably couple to activation of NFAT (Fig. 9A). These studies indicate that activation of NFAT pathways is not required for GPCR-mediated proliferative responses in general. However, each of the agonists tested can couple to activation of the SRE transcription factor, a downstream effector of Rho pathways (Fromm et al., 1997; Mao et al., 1998). Time courses for agonist coupling to SRE demonstrate that activation of SRE occurs within 2 h of application of each agonist. TFLLR and S1P coupling to SRE is maximal by 2 to 4 h and returns toward baseline, whereas LPA coupling to SRE is sustained out to 12 h (Fig. 9B).

Discussion

In the present study, we have examined the roles for PAR-1, LPA, and S1P receptors in initiating proliferative responses in murine astrocytes. Because activators of these receptors can enter the brain during trauma or ischemia, we have focused on the relationship between the underlying signaling pathways that are recruited by these receptors and astrocyte proliferation. The most important findings of this study are that activators of each of these receptor classes can induce short-term astrogliosis in vivo, and the recruitment of the MAPK pathway through Gi/o is essential for coupling each of these receptors to astrocyte proliferation. In addition, studies with pharmacological inhibitors suggest that activation of Rho pathways also plays a role in regulating proliferative responses for all three receptor classes that is independent of MAPK signaling cascades. Whereas previous reports have described varying aspects of PAR- or LPL-receptor mediated effects on astrocyte proliferation in vitro (Keller et al., 1997; Ramakers and Moolenaar, 1998; Tabuchi et al., 2000; Pebay et al., 2001; Steiner et al., 2002; Wang et al., 2002a; Yamagata et al., 2003), in this study we have sought to link in vivo effects of PAR-1, LPA, and S1P on reactive astrogliosis with shared signaling pathways for these receptors that mediate proliferative responses in vitro.

PAR-1, LPA, and S1P Receptors Share Intracellular Signaling Pathways. Although multiple PAR mRNAs are present in cultured astrocytes (Fig. 3A), previous studies have determined that PAR-1 plays a major role in activating proliferative responses in astrocytes, whereas PAR-3 and

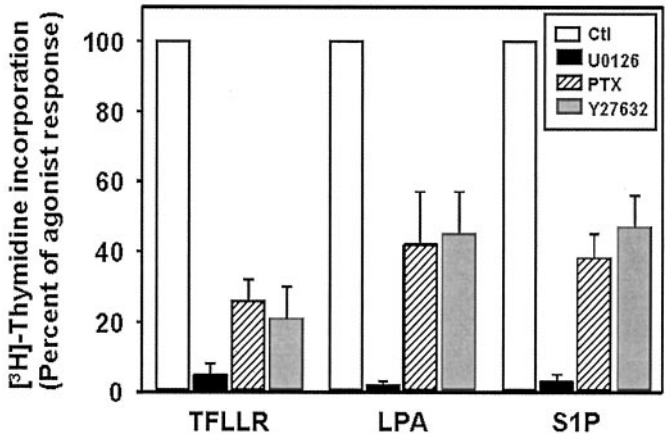

Fig. 8. Effect of signaling inhibitors on TFLLR, LPA, and S1P coupling to proliferation. Astrocytes were serum-starved for 24 h to arrest the growth of cells. Cells were treated with vehicle alone (Ctl), 10 μM U0126 (MEK inhibitor), 100 ng/ml PTX (Gi/o inhibitor), or 10 μM Y27632 (Rho kinase inhibitor) as described in the legend for Fig. 7 before treatment with buffer alone, 30 μM TFLLR, 30 μM LPA, or 10 μM S1P for an additional 24 h. [^{3}H]Thymidine was added for the final 2 h before recovery of [^{3}H]thymidine in the acid-insoluble material. Responses in the presence of inhibitor are reported as a percentage of response obtained in the absence of inhibitor [(agonist plus inhibitor in cpm − inhibitor alone in cpm)/(agonist alone in cpm − vehicle alone in cpm) × 100]. [^{3}H]Thymidine incorporation in cpm under control conditions for each inhibitor were for U0126: vehicle alone, 743 ± 468; U0126 alone, 70 ± 12; for PTX: vehicle alone, 1087 ± 216; PTX alone, 1055 ± 273; for Y27632: vehicle alone, 925 ± 239; Y27632 alone, 514 ± 91. n = 3 to 5.

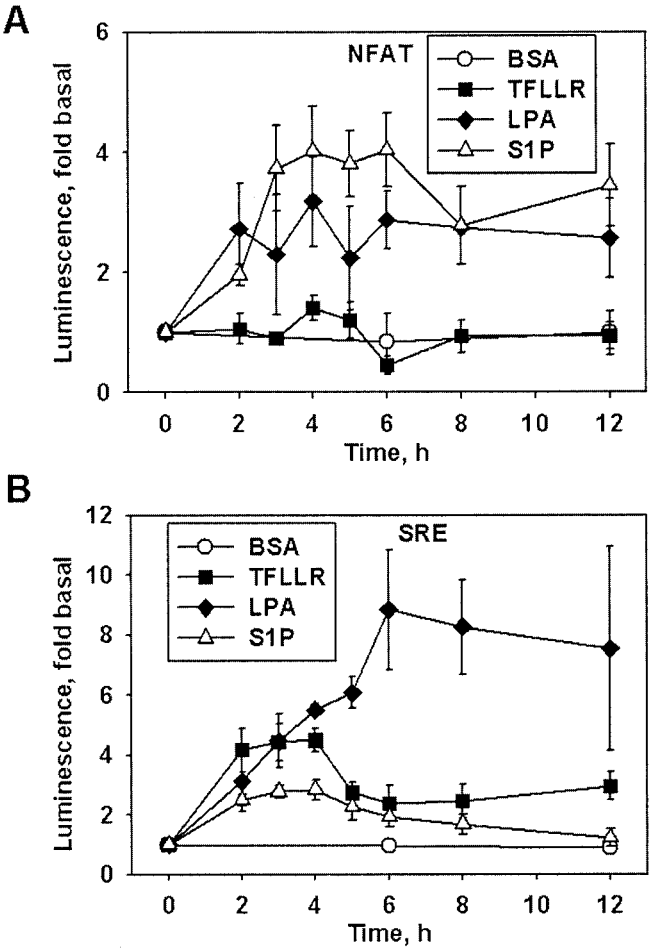

Fig. 9. PAR-1, LPA, and S1P coupling to NFAT or SRE transcription factors. Luciferase reporters were designed in a retroviral vector and astrocytes were infected with virus encoding either an NFAT binding element or an SRE binding element upstream of luciferase (see *Materials and Methods*). Infected astrocytes were serum-starved for 24 h before application of vehicle alone (BSA), 100 μM TFLLR, 30 μM LPA, or 10 μM S1P for the times shown. Luciferase activity in lysates was determined by luminescence. Luminescence obtained in lysates of mock-infected cells (approximately 25 units) were subtracted from the lysates of luciferase reporter-infected cells. Data are normalized to basal luminescence (22 ± 6 units for NFAT and 330 ± 180 units for SRE above mock-infected lysate) and are mean ± S.E.M. from three independent experiments.

PAR-4 are not mitogenic; activation of the thrombin-insensitive trypsin receptor PAR-2 initiates a moderate proliferative response (Wang et al., 2002a). Therefore, the present studies have focused on PAR-1 signaling events using a selective peptide activator (TFLLR) of PAR-1 that mimics the new N terminus revealed by thrombin cleavage at Arg_{41}. Dose-response curves for TFLLR reveal that the concentrations that induce relatively modest changes in upstream signaling events (phosphoinositide turnover) can induce robust downstream signaling in vitro (proliferation). This result is predicted by amplification of signaling pathways that is often evident on downstream effects. In contrast, higher doses of LPA and S1P were required to induce proliferation in vitro compared with doses required for phosphoinositide turnover (Fig. 5). These results seem to uncouple LPL-induced proliferative responses from phosphoinositide turnover. In support of this idea, treatment of astrocytes with a selective inhibitor of PKC also had no effect on agonist-mediated cell proliferation in culture. These data support previous results demonstrating that Gi/o proteins are important for astrocyte proliferation induced by S1P but activation of PKC is not (Pebay et al., 2001). Because phosphoinositide turnover is mediated through activation of Gq/11, these results suggest that the LPA- and S1P-mediated effects on proliferation are mediated by Gi/o and/or G12 pathways or are receptor-independent (Van Brocklyn et al., 1998; Spiegel and Milstien, 2000; Hooks et al., 2001). However, several alternate explanations of these data exist. For example, the dose-response relationship for phosphoinositide turnover induced by LPA or S1P occurs over a span of greater than 2 log and is better predicted by a variable slope fit versus a one-site fit (for LPA, $R^2 = 0.98$ versus 0.96; for S1P, $R^2 = 0.94$ versus 0.91 for variable slope versus one-site, respectively) with Hill slopes of less than 1. The identification of multiple mRNAs for different receptor subtypes is consistent with the idea that multiple LPA or S1P receptor subtypes mediate phosphoinositide turnover; perhaps only the low-affinity subset of those receptors is involved in proliferative responses. In addition, LPA and S1P may not be stable over the prolonged incubation times required for proliferative responses (24 h). Although we verified that LPA and S1P are biologically active while stored in aqueous 0.1% BSA after 24 h, the stability of these lipids in the presence of astrocytes is unknown. Therefore, the doses added to initiate these experiments may be much higher than the effective doses required for proliferation.

Similar to TFLLR, LPA and S1P both couple to the activation of the ERK1/2 and SAPK/JNK members of the MAPK family, and inhibition of these pathways with the MEK inhibitor U0126 completely inhibits proliferative responses to these agonists. These findings indicate an important role for ERK1/2 activation in the proliferation of astrocytes. Consistent with this idea, TFLLR and LPA each induced a sustained activation of ERK1/2 and a robust stimulation of [^{3}H]thymidine uptake, whereas S1P induced a transient activation of ERK1/2 (peak within 5 min) and a considerably more modest uptake of [^{3}H]thymidine. Together, these findings suggest that sustained activation of MAPK pathways may underlie proliferation of astrocytes. The coupling of TFLLR, LPA, and S1P to MAPK and proliferation can be further dissected by pharmacological inhibitors of the intermediary signaling pathways. PTX and Y27632 were used to define roles for Gi/o and Rho pathways, respectively, in these processes. Rho pathways are stimulated by G13-mediated activation of p115RhoGEF (Hart et al., 1998; Kozasa et al., 1998), although recent studies demonstrate that Rho pathways are also activated by Gq/11 independent of G12/13 (Sagi et al., 2001; Vogt et al., 2003). Although our studies did not directly measure activation of Rho in astrocytes, partial blockade of thymidine uptake by Y27632 indicates Rho involvement in astrocyte proliferation. Whereas both PTX and Y27632 disrupt proliferation, the coupling of these agents to MAPKs is sensitive only to PTX but not Y27632. Taken together, these studies suggest a similar mechanism of action for TFLLR, LPA, and S1P in coupling to MAPK that involves a Gi/o-dependent pathway. Although Y27632 attenuated proliferative responses, this agent had no effect on the capacity of TFLLR, LPA, or S1P to initiate MAPK cascades. Therefore, the involvement of Rho pathways in mediating proliferative responses seems to be independent of effects on the MAPK pathway (Fig. 10).

PAR-1, LPA, and S1P Receptors Regulate Transcription in Astrocytes. Signaling pathways can mediate global cellular changes via activation of transcription factors that regulate gene expression. Two such transcription factors that can be activated by Gi/o, Gq/11, or G12 signaling pathways are NFAT and SRE. NFAT is activated by the phosphatase calcineurin, which is in turn sensitive to changes in intracellular Ca^{2+}. Therefore, activation of PLC pathways, as well as other Ca^{2+} regulatory pathways, can induce activation of NFAT, which can bind DNA in cooperation with nuclear partners such as AP-1, a downstream component of MAPK cascades (Macian et al., 2001). NFAT has been shown to be involved in the regulation of a number of cellular events including proliferative responses in myocytes and endothelial cells (Horsley et al., 2001; Horsley and Pavlath, 2002; Johnson et al., 2003). Similar to NFAT, transcription through SRE is regulated by the cooperative binding of multiple transcription factors. SRE is bound by a ternary complex consisting of the serum response factor and cofactors such as Elk-1, a downstream component of MAPK cascades (Yordy and Muise-Helmericks, 2000). GPCRs have previously been shown to couple to activation of SRE through Rho-dependent mechanisms. Guanine-nucleotide exchange factors for Rho can be activated by both G12- and Gq/11-dependent path-

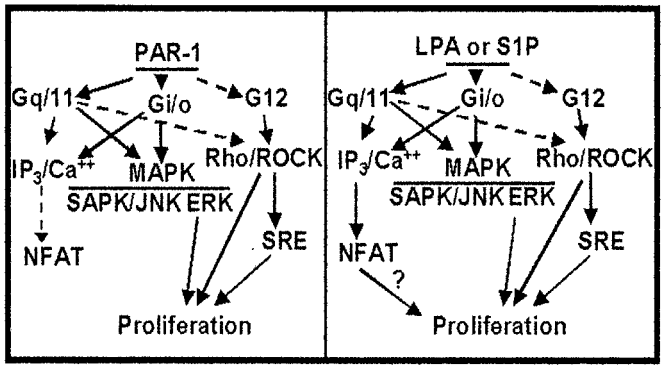

Fig. 10. Schematic representation of PAR-1, LPA, and S1P receptor signaling in astrocytes. Diagram depicts the deduced G protein signaling pathways and downstream effectors recruited by PAR-1, LPA, and S1P receptors based on functional and pharmacological data obtained in this study. The dashed lines indicate pathways that have not been clearly established in astrocytes. PAR-1–mediated Gq activation of MAPK pathways was demonstrated by Wang et al. (2002a). ROCK, Rho kinase.

ways (Fromm et al., 1997; Chikumi et al., 2002; Booden et al., 2002). However, in NIH 3T3 fibroblasts, thrombin and LPA activate SRE in the absence of Gq/11 (Mao et al., 1998). Together, these studies identify NFAT as a downstream effector of Gq/11-PLC and MAPK pathways, whereas SRE is activated by Rho and MAPK pathways.

In our studies, NFAT activation was most robust in the presence of S1P followed by LPA. Although these results are internally consistent with the enhanced activity of S1P to stimulate PLC pathways (compare Figs. 4 and 9), TFLLR coupling to NFAT was not detectable despite TFLLR coupling to PLC. These findings identify a point of divergence in PAR-1 and LPL receptor signaling and raise the possibility that PAR-1 recruits a distinct pathway that serves to limit NFAT activation. All three of the agents activated transcription through SRE. In general, agonists that coupled more robustly to SRE (LPA and TFLLR) also had higher intrinsic activity for proliferative responses. Together, these data favor a role for SRE pathways as important components of mitogenic responses in astrocytes.

Conclusions

Gliotic scarring is an important but poorly understood feature of CNS injury. Our data show that TFLLR, LPA, and S1P can all induce astrogliosis in vivo and provide comparative mechanistic insight into the intracellular pathways that couple the actions of these agents at their respective GPCRs to astrocyte proliferation. We show that each of these agents activates signaling pathways downstream of Gi/o, Gq/11, and possibly G12/13 in concert and that activation of MAPK cascades through Gi/o-linked pathways plays a central role in regulating PAR-1- and LPL-mediated proliferation. Rho-SRE pathways also play an important role in regulating proliferation independent of MAPK. Together, these studies emphasize the similar intracellular mechanisms for PAR-1 and LPL receptor-mediated activation of proliferation and begin to identify the specific role for transcriptional activation in this process.

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