Molecular and Cellular Neuroscience 50 (2012) 21–34

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Lysophosphatidic acid induces neurite branch formation through LPA_3

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ARTICLE INFO

Article history:
Received 3 October 2011
Revised 3 February 2012
Accepted 14 March 2012
Available online 22 March 2012

Keywords:
Lysophosphatidic acid
LPA_3
Rnd2
G_q
Neurite branching
Hippocampal neuron

ABSTRACT

Although neurite branching is crucial for neuronal network formation after birth, its underlying mechanisms remain unclear. Here, we demonstrate that lysophosphatidic acid (LPA) stimulates neurite branching through a novel signaling pathway. Treatment of neuronal cell lines with LPA resulted in neurite branch formation when LPA_3 receptor was introduced. The effects of LPA were blocked by inhibition of G_q signaling. Furthermore, expression of inhibitory mutants of the small GTPase Rnd2/Rho7 or an Rnd2 effector rapostlin abolished LPA_3-mediated neurite branching. The LPA_3 agonist 2(S)-OMPT or LPA also induced axonal branch formation in hippocampal neurons, which was blocked by G_q and Rnd2 pathway inhibition or LPA_3 knockdown. These findings suggest that the novel signaling pathway involving LPA_3, G_q, and Rnd2 may play an important role in neuronal network formation.

Introduction

Sprouting, extension, and branching of neurites are essential to complex neuronal network formation during nervous system development. Over the past few decades, many neurotrophic factors and guidance molecules have been identified and found to enhance neurite sprouting and elongation and to regulate orientation (Baird, 1994; Barbacid, 1995; Brose and Tessier-Lavigne, 2000; Culotti and Merz, 1998; Polleux and Snider, 2010; Quinn and Wadsworth, 2008). Likewise, several extracellular signaling molecules inducing neurite branch formation or arborization have been reported (Cheng et al., 2003; Dent et al., 2004; Kvachnina et al., 2005; Szebenyi et al., 2001). These include basic fibroblast growth factor (bFGF) and serotonin. However, the intracellular mechanisms involved, from receptor activation to neurite branching, remain unclear.

Branch formation is an alteration of neurite morphology that requires rearrangement of the cytoskeleton. The small GTPase family, including RhoA, Rac, and Cdc42, consists of signal transducers that link extracellular signals to the cytoskeleton and are known to regulate neuritogenesis and neurite outgrowth through actin reorganization (Govek et al., 2005; Watabe-Uchida et al., 2006). Recently, a unique GTPase, Rnd2/Rho7, the functions of which are not yet fully understood (Riou et al., 2010), was found to be involved in neurite branching in pheochromocytoma12 (PC12) cells and hippocampal neurons (Fujita et al., 2002; Kakimoto et al., 2004). Activation of Rnd2 relays signals to rapostlin (formin-binding protein 17/FBP17), an Rnd2-interacting protein, followed by stimulation of neural Wiskott–Aldrich syndrome protein (N-WASP). N-WASP then induces actin polymerization, resulting in the alteration of neurite morphology leading to branching formation. However, the extracellular signals linked to Rnd2 activation remain unclear.

Lysophosphatidic acid (LPA) is an extracellular signaling lipid that regulates cell morphology through G protein-coupled LPA receptors (Fukushima, 2004; Fukushima et al., 2001; Ishii et al., 2004). Six LPA receptor subtypes (*Lpar1–Lpar6*) have been identified to date (Ishii et al., 2009; Noguchi et al., 2009). *Lpar1* is expressed in cortical neuroepithelial cells during the embryonic period, *Lpar2* expression occurs widely in the embryonic brain and disappears around 1 postnatal week, and *Lpar3* expression in brain begins at the late embryonic period and continues up until 1 postnatal week (Contos et al., 2000; Hecht et al., 1996; McGiffert et al., 2002). Expression of *Lpar4* and *Lpar5* in embryonic brains has also been demonstrated (Lee et al., 2006, 2007). These observations suggest that LPA receptor-mediated signaling plays an important role in brain development.

To address the role of LPA receptor-mediated signaling in the nervous system, LPA-dependent cellular responses have been investigated extensively in neuronal cells (Fukushima, 2004; Fukushima and Morita, 2006; Ishii et al., 2004, 2009; Noguchi et al., 2009; Ueda, 2011; Yamane et al., 2010). All LPA receptor subtypes except LPA_3 have been reported to mediate LPA-induced neurite retraction and growth cone collapse (Fukushima, 2004; Ishii et al., 2009; Noguchi et al., 2009). On the

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doi:10.1016/j.mcn.2012.03.006

contrary, LPA_3 failed to induce these responses (Ishii et al., 2000). Thus, LPA_3 receptors may have a distinct role in the developing nervous system from that of other LPA receptor subtypes. In this study, we show that LPA_3 stimulation increases axon branching through the activation of G_q subunit and Rnd2.

Results

Effects of LPA in PC12 cells exogenously expressing LPA_1, LPA_2 or LPA_3

Because PC12 cells differentiate into sympathetic neuron-like cells in the presence of neurotrophic factors, they have been widely used to examine the molecular machinery of neurite outgrowth and neuronal differentiation. RT-PCR analyses revealed that PC12 cells endogenously expressed *Lpar1*, *Lpar2*, and *Lpar3* genes (Fig. 1A). This expression was essentially unaltered by nerve growth factor (NGF) treatment. By contrast, no *Lpar4* or *Lpar5* expression was observed in naïve or NGF-treated PC12 cells.

To examine whether LPA receptor overexpression in PC12 cells influenced NGF-induced neurite outgrowth and morphology, PC12 cells were infected with retroviruses co-expressing LPA receptor and green fluorescent protein (GFP) (Fukushima et al., 2002a), and were cultured in the absence or presence of LPA and/or NGF under serum-free conditions. Few neurite-bearing cells were observed in control virus-infected cells in the absence of NGF (data not shown). NGF treatment increased the percentage of neurite-bearing cells by 65% (Figs. 1B and C). LPA exposure during the culture period slightly reduced this percentage, possibly due to the suppression of neurite outgrowth through endogenous LPA_1 or LPA_2 receptor. Overexpression of LPA_1 in PC12 cells completely inhibited NGF-induced neurite outgrowth, independent of the presence of LPA (Figs. 1B and C), suggesting that exogenously overexpressed LPA_1 was constitutively activated, as reported (Shano et al., 2008; Yoshida and Ueda, 1999). LPA_2-expressing cells also showed reduced responses to NGF, both in the reduction in the percentage of neurite-bearing cells and in the shortening of neurites (Figs. 1B and C). This result suggested that exogenous LPA_2 was also intrinsically activated at some lower level than LPA_1. NGF-induced neurite outgrowth in LPA_2-expressing cells was partially suppressed by treatment with LPA (Fig. 1C). By contrast, approximately 10% LPA_3-expressing cells showed neurite outgrowth independent of NGF and LPA (Fig. 1C). However, when NGF was added, the percentage of neurite-bearing cells was comparable to that of control cells, and LPA inhibition of neurite outgrowth was similarly observed (Fig. 1C). This inhibition may be explained by the activation of endogenous LPA_1 and LPA_2 by LPA.

Interestingly, LPA_3-expressing cells exhibited more branching points in the presence of NGF and LPA (Fig. 1B). About 50% of control virus-infected cells possessed one or more branching points, and the ratio was unchanged by LPA treatment (Fig. 1D). By contrast, more than 90% of LPA_3-expressing cells produced neurite branching in response to LPA exposure (Fig. 1D), and the number of branching points per LPA_3-expressing cell increased by 3-fold (Fig. 1E). We also determined the number of neurites per neurite-bearing cell in NGF-treated control and LPA_3-expressing cells. On average, nearly four neurites extended from each control cell body, which was unchanged by LPA treatment (Fig. 1F). LPA_3-expressing neurite-bearing cells produced about five neurites each in response to LPA. Thus, LPA_3 activation markedly stimulated branching and also significantly increased neurite formation during NGF-induced neuronal differentiation.

Heterologous expression of LPA_3 in B103 neuroblastoma cells

We previously reported that rat B103 neuroblastoma cells did not respond to LPA with any morphological changes or activation of signaling pathways, and that transient, heterologous LPA_3 expression in B103 cells resulted in G protein activation (Fukushima et al., 1998; Ishii et al., 2000). Therefore, clonal cells co-expressing LPA_3 and GFP were established to examine neurites branch formation (Figs. 2A, B). After 24-h serum starvation, the number of branching points in the control clone (AB2-1bf) was slightly increased over the culture period (0.50 ± 0.09 neurites/cell before starvation, 0.88 ± 0.14 neurites/cell after starvation, mean $\pm$ s.e.m., 200 cells from four experiments). LPA exposure for an additional 18 h had no effect on branching (Figs. 2A, C). The LPA_3-expressing clone (designated lpa3-3-2) also demonstrated increased branching points after serum starvation (0.68 ± 0.12 neurites/cell before starvation, 1.22 ± 0.14 neurites/cell after starvation, mean $\pm$ s.e.m., 200 cells from four experiments). When LPA was further added to lpa3-3-2 cells for an additional 18 h, a significant increase in branching points was observed (Figs. 2A, C). An LPA_3-specific agonist, 1-oleoyl-2-O-methyl-rac-glycerophosphothionate (2(S)-OMPT) (Hasegawa et al., 2003), also enhanced neurite branching formation, whereas an LPA_1/LPA_3 antagonist, dioctylglycerol pyrophosphate (DGPP) (Fischer et al., 2001), inhibited the effect of LPA and 2(S)-OMPT (Fig. 2C). The same effects were observed in other LPA_3-expressing B103 clones or B103 cells transiently infected with LPA_3-expressing viruses, indicating that they were not due to clonal variation (Fig. 2D). A significant increase in branching points was observed at 4 h after exposure to LPA, and this effect peaked at 8 h after LPA addition (Fig. 2E).

Signaling pathways involved in LPA_3-mediated branching formation

We next examined the signaling pathways involved in LPA_3-mediated branch formation. LPA_3 is known to couple to $G_{i/o}$ and G_q, but not G_s or $G_{12/13}$ subunits, in B103 cells (Ishii et al., 2000). When lpa3-3-2 cells were transfected with an RGS4 plasmid that inhibited both $G_{\alpha i}$ and $G_{\alpha q}$ (Shano et al., 2008), LPA-induced neurite branch formation was inhibited (Figs. 3A and B). Expression of the carboxyl terminus of GRK2ct, a $G_{\beta\gamma}$-sequestrating peptide, or p115RGS, a RGS domain of p115RhoGEF inhibiting $G_{12/13}$ (Shano et al., 2008), failed to change the effects of LPA (Figs. 3A and B). To separate the G_i and G_q pathways, the $G_{i/o}$ inhibitor pertussis toxin (PTX) and G_q inhibitor YM-254890 were used. PTX treatment of lpa3-3-2 cells showed no effect on LPA-induced neurite branching (Figs. 3C and D). By contrast, YM-254890 inhibited the effects of LPA (Figs. 3C and D), indicating the involvement of G_q in LPA_3-mediated neurite branching. A major effector of G_q is phospholipase C (PLC), which generates inositol 1,4,5-trisphosphates and diacylglycerol. When lpa3-3-2 cells were exposed to the PLC inhibitor U-73122, LPA failed to stimulate neurite branching (Figs. 3C and D). An inactive compound, U-73343, was ineffective. Taken together, these results suggested that LPA_3-induced stimulation of G_q-PLC led to neurite branch formation.

The small GTPase family has been shown to regulate neurite morphology and outgrowth through interactions with cytoskeleton-associated proteins (Govek et al., 2005; Watabe-Uchida et al., 2006) including Rho, Rac and Cdc42, all of which consist of small subfamilies. Rnd2 is a Rho family member expressed in fetal and adult brain(Nobes et al., 1998), and interactions between Rnd2 and rapostlin have been shown to stimulate neurite branching in PC12 cells (Fujita et al., 2002), which resembled LPA_3-mediated neurite branch formation. Because both Rnd2 and rapostlin were expressed in lpa3-3-2 and PC12 cells (Fig. 4A), Rnd2/rapostlin pathway involvement in LPA_3-mediated neurite branch formation was next investigated.

Like other small GTPase proteins, the amino acid substitution of Thr21 to Asn, or Ala16 to Val, in Rnd2 produces dominant-negative Rnd2N21 or constitutively active Rnd2V16 forms, respectively (Fujita et al., 2002). We introduced these mutants into lpa3-3-2 cells and examined the effect of LPA on neurite branch formation. Rnd2N21 expression suppressed LPA-induced neurite branching, whereas Rnd2V16 expression resulted in marked branch formation without LPA stimulation (Figs. 4B, C). The amino-terminal or carboxyl-terminal deletion mutants of rapostlin, rapostlin-lN or rapostlinΔSH3, respectively, which have been shown to inhibit Rnd2V16-induced neurite branching formation