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Role of iPLA₂ in the regulation of Src trafficking and microglia chemotaxis

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Abstract

Microglia are immune effector cells in the CNS and their activation, migration, and proliferation play crucial roles in brain injuries and diseases. We examined the role of iPLA₂ in the regulation of microglia chemotaxis toward ADP. Inhibition of iPLA₂ by BEL or iPLA₂ knock-down exerted a significant inhibition on PI3K activation and chemotaxis. Further examination revealed that iPLA₂ knock-down abrogated Src activation which is required for PI3K activation and chemotaxis. Colocalization studies demonstrated that cSrc-GFP was retained in the endosomal recycling compartment (ERC) in iPLA₂ knock-down cells, but addition of arachidonic acid (AA) could restore cSrc trafficking to the plasma membrane by allowing the formation/release of recycling endosomes associated with cSrc-GFP. Using BODIPY-AA, we showed that AA is selectively enriched in recycling endosomes. These results suggest that AA is required for the cSrc trafficking to the plasma membrane by controlling the formation/release of recycling endosomes from the ERC.

Keywords

Phospholipase A₂; Arachidonic acid; chemotaxis; microglia; cSrc; recycling endosome

Introduction

The directed movement of cells in response to chemical stimulation is involved in many physiological and disease processes. In order to sense and respond to concentration gradients, cells must obtain an internal signaling polarity that is aligned with the concentration gradient. When *Dictyostelium* and many other cell types are exposed to a chemoattractant gradient, phosphoinositol-3-kinase (PI3K) is localized selectively to the leading edge membrane, allowing for the spatially restricted production of 3'-phosphoinositides (3'-PI's). Local production of 3'-PIs and F-actin polymerization overlap at the front of migrating *Dictyostelium*, neutrophils, and fibroblasts (1-3), and a positive feedback loop between PI3K and F-actin polymerization regulated by Rho GTPase has been suggested in previous studies (4, 5). The spatially and mutually exclusive localization of the PI3K and phosphatase PTEN provides a mechanism to sharply regulate the membrane

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localization of this second messenger, creating a steep intracellular signaling gradient. As a result, 3'-PI signaling has been considered to be essential for the regulation of chemotaxis and cell polarity. Recent evidence also point that additional pathways act in parallel. A genetic screen in *Dictyostelium discoideum* to identify redundant pathways revealed that loss of Phospholipase A₂ (PLA₂) did not alter PI(3,4,5)P(3) regulation, but chemotaxis became sensitive to reductions in PI3K activity (6). Strong chemotaxis defects are observed only when both the PI3K and PLA₂ pathways are disrupted. Likewise, pharmacological inhibition of either PI3-kinase or PLA₂ inhibits chemotaxis in shallow cAMP gradients, whereas both enzymes must be inhibited to prevent chemotaxis in steep cAMP gradients, suggesting that PI3-kinase and PLA₂ are two redundant mediators of chemotaxis(7).

PLA₂s catalyze the hydrolysis of the sn-2 ester bond of cellular glycerophospholipids, generating lysophospholipids and free fatty acids. PLA₂s can be classified into three main types; the secretory PLA₂ (sPLA₂), the cytosolic Ca²⁺-dependent PLA₂ (cPLA₂) and the intracellular Ca²⁺-independent PLA₂ (iPLA₂)(8, 9). sPLA₂ is a relatively small (14 kDa) enzyme and they do not manifest significant fatty acid selectivity *in vitro*, but require millimolar Ca²⁺ for catalytic activity. The cPLA₂ is a 85 kDa enzyme with a specificity for phospholipids containing arachidonic acid. It requires nanomolar to micromolar concentrations for its activity and has a C2 domain, allowing its translocation to membranes in response to an increase in intracellular Ca²⁺. Currently, a few members of the iPLA₂ family have been identified and these iPLA₂ isoforms share sensitivity to 4-bromo-enol lactone (BEL), an irreversible inhibitor (9,10). Two isoforms, group VIA iPLA₂ (also called iPLA₂β) and group VIB iPLA₂ (also called iPLA₂γ), are abundantly expressed in mammals(11). iPLA₂β is an 85-kDa protein that does not require calcium for its catalytic activity. A unique feature of the iPLA₂β is that it contains eight ankyrin motifs at the N-terminus.

Recent studies showed that reduced expression of iPLA₂β or cPLA₂ with antisense oligodeoxyribonucleotide leads to a significant defect of monocyte chemotaxis toward monocyte chemoattractant protein-1 (MCP-1), mainly due to the reduction of cell speed(12). This chemotaxis defect was restored if cells were treated with lysophosphatidic acid (LPA) or arachidonic acid (AA), yet both enzymes are required for monocyte chemotaxis to MCP-1. In addition, iPLA₂β appeared to regulate directionality and actin polymerization(13). Results from studies on chemotaxis of *Dictyostelium* or monocytes clearly indicate that PLA₂ plays an important role in the regulation of chemotaxis, but mechanistic details of how PLA₂ activity is required for the regulation of chemotaxis are not clear. In this study, we report that iPLA₂β activity is required for the regulation of microglia chemotaxis via controlling the recycling endosome-mediated trafficking of Src to the plasma membrane.

Results

iPLA₂ activity is required for the activation of PI3K and chemotaxis

Previous studies showed that extracellular ATP or ADP could induce PI3 kinase activation and chemotaxis of microglia via the Gi/o-coupled P₂Y₁₂ receptor (P₂Y₁₂R)(14-16) and that ADP stimulation significantly increased the level of Akt phosphorylation at Thr³⁰⁸ that can be blocked by LY294002(general PI3K inhibitor), but not by AS604850(PI3Kγ-specific inhibitor)(17). To investigate the role of iPLA₂ in the regulation of PI3K activity and microglia chemotaxis, we examined Akt phosphorylation at Thr³⁰⁸ upon ADP stimulation in microglia cells treated with bromoenol lactone (BEL), a highly selective iPLA₂ inhibitor (9) (Fig. 1A). Phosphorylation of Akt at Thr³⁰⁸ was significantly inhibited by BEL, indicating iPLA₂ activity is required for the activation of PI3K. Inhibition of Akt phosphorylation by BEL can be rescued by the addition of 30μM arachidonic acid (AA) to the medium.

Surprisingly, AA alone, without ADP stimulation, can elicit activation of Akt, suggesting that iPLA₂ activity or AA plays an important role in the regulation of PI3K. To examine the effect of iPLA₂ inhibition on chemotaxis, microglia chemotaxis was assessed using a transwell migration assay. In the presence of BEL, microglia did not migrate toward the lower chamber containing 100 μ M ADP (Fig. 1B), presumably due to inability to activate PI3K as LY294002 also significantly blocked the chemotaxis. One can expect the activity of iPLA₂ to be regulated upon ADP stimulation if iPLA₂ activity is required for the regulation of PI3 kinase. To determine if there is an increase of AA production upon ADP stimulation and to assess the contribution of iPLA₂ to the increase of AA, we examined the level of free AA in cells by using MeOH/DCM extraction after steady-state labeling with ³H-arachidonic acid for 6 hours (Fig. 1C). Upon ADP stimulation, there is roughly a 75% increase of free AA in cells. This increase can be effectively blocked by pretreatment of cells with BEL, indicating that iPLA₂ activity can be upregulated upon P₂Y₁₂R activation. Free AA can be enzymatically converted to numerous bioactive signaling molecules via the cyclooxygenase (COX) and lipoxygenase (LOX). To test a possibility that metabolite of AA could act indirectly as signaling molecules to regulate PI3K, we examined Akt phosphorylation upon ADP stimulation in cells treated with 10 μ M Indomethacin (a non-selective COX inhibitor), 10 μ M NS-398 (a selective COX-2 inhibitor) for 30 min or 10 μ M Nordihydroguaiaretic Acid (a non-selective lipoxygenase inhibitor), 1 μ M Baicalein (a selective 12-lipoxygenase inhibitor) for 20min (Fig. 1D). None of these inhibitors significantly blocked Akt phosphorylation upon ADP stimulation, indicating that PI3K regulation is not mediated by metabolites of AA. iPLA₂ exhibits no apparent substrate specificity for phospholipids containing arachidonic acid(8, 9). Thus, it is plausible that other fatty acids might have a similar role in the regulation of PI3K. We tested if various fatty acids with different lengths and number of double bonds could activate Akt phosphorylation like AA (Fig. 1E). Oleic acid (18:1), Dihomo- γ -linolenic acid (20:3), Linoleic acid (18:2), Eicosapentaenoic acid (20:5) were tested but no significant activity to stimulate Akt phosphorylation was detected except oleic acid which showed moderate Akt activation. To exclude the possibility of nonspecific inhibition of other types of PLA₂ by BEL, we tested if Akt phosphorylation was inhibited in iPLA₂ β knock-down (iPLA₂ β -KD) cells. Similar to BEL-treated cells, iPLA₂ β -KD cells did not show the increase of Akt phosphorylation upon ADP stimulation and AA can rescue this defect (Fig. 1F). Expression of human iPLA₂ β in iPLA₂ β -KD cells rescued Akt phosphorylation upon ADP stimulation (data not shown), ruling out off-target effects of shRNA. These results demonstrate a unique and specific role of AA in the regulation of PI3K activation upon P₂Y₁₂R activation by ADP.

Src activity is required for PI3K activation upon ADP stimulation

To examine mechanistic details of the inhibition of PI3K activation by BEL or iPLA₂ β knock-down, we first examined Ras activation since Ras has been demonstrated to activate PI3K by interacting directly with the catalytic subunit of PI3K through the Ras effector site(18). A pull-down assay with GST-Raf/RBD did not show any significant activation of Ras upon P₂Y₁₂R activation (not shown). Activation of PI3-kinase by Src-family kinases through binding of the p85 subunit to the Src-SH3 domain has also been reported(19) and we tested if Src activity is required for PI3K activation. A pharmacological inhibitor of the Src family tyrosine kinases, PP2, significantly inhibited ADP-induced Akt phosphorylation on Thr³⁰⁸ in microglia (Fig. 2A), indicating that Src kinase activity is required for the activation of PI3K. Interestingly, Akt phosphorylation was completely abrogated in cells treated with both PP2 and BEL. Since PP2 could inhibit multiple Src family kinases, we used knock-down experiments to identify a member of Src family kinases responsible for the activation of PI3K. Knock-down of cSrc expression with shRNA significantly reduced Akt phosphorylation while Fyn knock-down had no impact on Akt phosphorylation (Fig. 2B), indicating cSrc is involved in the PI3K activation. Chemotaxis of cells treated with

BEL or iPLA₂β-KD cells was significantly inhibited and expression of human iPLA₂β-GFP restored chemotactic ability of cells while GFP alone did not (Fig. 2C). PP2 also abrogated microglia chemotaxis effectively. Src activation requires carboxy-terminal Tyr⁵³⁰ (Tyr⁵³⁴ for mouse) dephosphorylation and subsequent kinase domain Tyr⁴¹⁸ (Tyr⁴²³ for mouse) autophosphorylation (20). Consistent with this, Src was rapidly dephosphorylated at Tyr⁵³⁴ and BEL blocked Src dephosphorylation at this site (Fig. 2D). Src activity appears to be at a maximum level in 2 min after ADP stimulation while Akt phosphorylation peaks at 5 min. Moreover, ADP-induced Src phosphorylation at Tyr⁴²³ was not inhibited by the PI3-kinase inhibitor, LY294002. These results indicate that Src activity is required for the regulation of PI3K activation and chemotaxis and that Src signaling is upstream of the activation of PI3-kinase/Akt pathway. Similar to Akt phosphorylation, inhibition of iPLA₂ activity by BEL significantly reduced Src activation upon ADP stimulation and AA could rescue this inhibition (Fig. 2E).

Src activity is required for the formation of membrane protrusions via phosphorylation of p130Cas recruiting Dock180 and ELMO1 and for focal adhesions and membrane ruffles via phosphorylation of paxillin(21). We examined if inhibition of Src activity by BEL has an impact on focal adhesions and membrane ruffles. In our previous study(17), we demonstrated that the number of focal adhesions was markedly increased up to three times and the size was also increased upon ADP stimulation. In contrast to control cells, there was no increase of focal adhesion number and size in cells treated with BEL (Fig. 3A), indicating that iPLA₂ activity is required for the regulation of focal adhesions presumably via the regulation of Src activity. To elucidate how dynamic regulation of ruffle formation is altered by iPLA₂ inhibition, we employed a video microscopy over a 15 min period after ADP treatment followed by kymography analysis. Both frequency and distance of membrane protrusion and retraction are greatly enhanced after ADP treatment (Fig. 3B). BEL treatment almost abolished ruffle formation in the absence or presence of ADP as the frequency of ruffle formation was significantly reduced by BEL. These results indicate that inhibition of Src activity by BEL has significant negative impact on the regulation of focal adhesion and ruffle formation, leading to inefficient chemotaxis.

Inhibition of Src translocation to plasma membrane in iPLA₂β-KD cells

In the absence of mitogenic stimuli, Src was shown to be localized at microtubule-associated endosomes around the nucleus. Upon stimulation, however, Src translocates to the cell periphery (22-24). We examined the subcellular localization of Src and pSrc/Tyr⁴²³ in cells treated with BEL or in iPLA₂β-KD cells using indirect immunofluorescence staining. Src is predominantly associated with vesicular structures in the perinuclear area and a low level of pSrc/Tyr⁴²³ staining was observed at the plasma membrane in control cells (Fig. 4A). Upon ADP stimulation, a large fraction of Src and pSrc was translocated and associated with the cortical membrane. This is consistent with the increase of Src phosphorylation at Tyr⁴²³ and translocation to the membrane upon stimulation. In contrast, translocation of Src and pSrc/Tyr⁴²³ upon stimulation is markedly reduced in cells treated with BEL. Most of Src and pSrc/Tyr⁴²³ staining were remained in the perinuclear area. Taken together, these observations suggest the presence of a defect in Src trafficking to the plasma membrane in BEL-treated cells. This defect in Src trafficking could be recovered by the addition of 30μM AA to cells in the presence of BEL, which leads to Src and pSrc staining on vesicles/tubules near the plasma membrane. In some cases, these vesicles appear to be on tubular networks (Fig. 4A). Quantification of fluorescence intensity of Src or pSrc staining at the plasma membrane clearly shows that inhibition of iPLA₂ by BEL effectively blocks Src trafficking to the plasma membrane and that exogenous AA can rescue this trafficking defect (Fig. 4B). The Src trafficking defect can also be observed in iPLA₂β-KD cells, and AA addition or expression of human iPLA₂β-GFP can rescue Src trafficking in these cells (Fig. 4C). Similar

to BEL-treated cells, AA addition to iPLA₂β-KD cells induces formation of numerous vesicles associated with cSrc-GFP that appear to be transported on a tubular network. We examined the movement of these vesicles in iPLA₂β-KD cells using a time-lapse videomicroscopy (Fig. 4D). Tracking of these vesicles in iPLA₂β-KD cells revealed random movement of vesicles. Upon addition of AA, vesicles showed directional movement toward the plasma membrane as shown in Figure 4D, indicating that AA is required for the Src trafficking. Directional movement of cSrc-GFP upon AA addition suggests that the Src trafficking defect in iPLA₂β-KD cells might result from defective microtubule organization. We examined cells treated with BEL or iPLA₂β-KD cells by immunofluorescence with anti-tubulin antibodies and found no obvious effects on microtubule number or distribution (not shown), suggesting that the Src trafficking defect is not caused by abnormal microtubule organization.

Src remains in endosomal recycling compartment in iPLA₂β-KD cells

In an attempt to identify the perinuclear compartment to which Src localizes at a steady state in BEL-treated cells, we examined the colocalization of Src with various subcellular organelle markers (Fig. 5A). Immunofluorescence staining of GM130 (a cis-Golgi marker(25)), fluorescence of Cerulean-HDEL (a ER marker), or LysoTracker staining did not show significant colocalization with Src staining or GFP-cSrc whereas TRITC-Wheat-germ Agglutinin (WGA, a TGN marker(26)) and mannose-6-phosphate receptor (MPR, a *trans*-Golgi and late endosome marker) showed moderate colocalization with Src. Overall, these results show that, at a steady state, the majority of Src protein is not associated with organelles from the exocytic pathway in microglia. Moreover, GM130 and WGA staining showed no sign of Golgi dispersion, suggesting that the Src trafficking defect is not a result of a morphological defect in the TGN. Since Src localization to the pericentriolar recycling endosome was previously observed(27, 28), we examined the possibility that Src could be associated with this compartment instead of the Golgi complex. We used two endosomal markers, Rab7 (a marker for early (EE) and late endosomes (LE)(29, 30)) and Rab11 (a marker for endosomal recycling compartment (ERC) and recycling endosome (RE) (31)). GFP-c-Src expressed in iPLA₂β-KD cells showed strong colocalization with both mCherry-Rab7 and -Rab11, but not with LysoTracker. This result suggests that a significant fraction of Src might be retained in an endosomal recycling compartment (ERC) in iPLA₂β-KD cells. To further examine if iPLA₂β activity or AA is required for the release of vesicles from the ERC and recycling back to the plasma membrane, we loaded cells with Alexa⁵⁹⁵-transferrin (Tf), a marker for ERC(32). At steady state, a majority of the internalized transferrin localized to the ERC in control cells (Fig. 5B). The ERC was mainly labeled with transferrin after 30 min of loading in control cells and many recycling endosomes were labeled with Tf after an hour. However, Tf was still accumulated in ERC in iPLA₂β-KD cells after an hour even though the amount of internalized transferrin was not significantly affected. This result indicates that recycling of Tf back to the plasma membrane was impaired in iPLA₂β-KD cells. The Tf that had accumulated in the ERC of iPLA₂β-KD cells showed strong colocalization with cSrc-GFP (Fig. 5C), indicating that both were retained at ERC.

Results from previous experiments strongly suggest that AA might be required for the formation/release of recycling endosomes from the ERC. To test this, we examined colocalization between cSrc-GFP and two markers, mCherry-Rab7 and -Rab11 before and after AA addition (Fig. 6A). In iPLA₂β-KD cells, cSrc-GFP, as well as Rab7 and Rab11, localize to the ERC. Upon addition of AA, smaller vesicles associated with GFP-c-Src and mCherry-Rab11, but not with Rab7, were formed. These results suggest that Src remains in the ERC in iPLA₂β-KD cells and that AA produced by iPLA₂ activity is required for the trafficking of GFP-c-Src from the ERC to recycling endosomes. To examine if there is a

systematic defect in trafficking when iPLA₂ activity is inhibited, we investigated the trafficking of two other proteins, vesicular stomatitis viral G protein (VSV-G) and H-Ras, which are known to be trafficked to the plasma membrane via secretory pathways (Fig. 6B and C). We used the temperature-sensitive mutant of VSV-G fused to YFP (VSVG-YFP) that is retained in the ER at 40°C, but trafficked to the Golgi complex and to the plasma membrane at 33°C (33). At 40°C, VSVG-YFP appears to be retained in the perinuclear area while Src was localized to the plasma membrane. Both Src and VSV-G-YFP were retained in the perinuclear region at 40°C in cells treated with BEL. Shifting the temperature back to 33°C rescued VSV-G-YFP trafficking to the membrane but not Src trafficking in BEL-treated cells, indicating that the trafficking defect caused by the inhibition of iPLA₂ activity is specific to Src. Similarly, GFP-H-Ras appeared to be on vesicles and trafficked normally in iPLA₂β-KD cells.

Selective enrichment of AA in recycling endosomes

Next we determined if AA accumulates in the ERC or recycling endosomes by examining the distribution of BODIPY-labeled AA. BODIPY-AA was synthesized using a carbodiimide-mediated conjugation reaction(34). The BODIPY moiety is covalently attached to the fatty acid at the carboxyl group using the activating compound 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC). Addition of BODIPY-AA to iPLA₂β-KD cells could induce Akt activation and cSrc-vesicle release from ERC (Fig. 7A and B), indicating that the biological activity of AA is not changed by the tag at least for the rescue of the Src trafficking defect in iPLA₂β-KD cells. Upon addition of the AA:BODIPY-AA mixture (10:1) to iPLA₂β-KD, BODIPY-AA was preferentially enriched at the ERC and Rab11-positive vesicles, but not with Rab7-positive vesicles or lysosomes (Fig. 7C). This is consistent with the idea that AA is required for the release of recycling endosomes from ERC.

Discussion

In the present study, we demonstrated that iPLA₂ activity is specifically required for the trafficking of c-Src to the plasma membrane. Results from studies on chemotaxis of *Dictyostelium* and monocytes clearly indicate that PLA₂ plays an important role for the regulation of chemotaxis. A genetic screen in *Dictyostelium discoideum* revealed that loss of iPLA₂ did not alter PI(3,4,5)P(3) regulation, but chemotaxis became sensitive to a reduction in PI3K activity (6). Partly redundant signaling cascades including PI3K and PLA₂ make chemotaxis in *D. discoideum* very robust because multiple signaling pathways must be deleted to obtain a strong reduction of chemotaxis(35). Recent studies showed that reduced expression of iPLA₂β or cPLA₂ with siRNA leads to a significant defect in monocyte chemotaxis toward monocyte chemoattractant protein-1 (MCP-1)(12). iPLA₂β appeared to regulate directionality and actin polymerization during monocyte chemotaxis(13). These studies clearly indicate that iPLA₂ plays an important role in the regulation of chemotaxis but mechanistic details of how PLA₂ activity is required for the regulation of chemotaxis was not known. Our study provides a further mechanistic insight and demonstrates that iPLA₂ activity is required for the trafficking of key signaling molecules including cSrc. Src family kinases (SFKs) have been identified to be essential for acute inflammatory responses by controlling the recruitment and activation of monocytes, macrophages, and neutrophils. Macrophages and monocytes with a lack of SFKs display marked alterations in cytoskeleton dynamics and polarization and their recruitment into inflammatory sites was significantly reduced(36). Src trafficking and activation appears to be linked as Src was shown to be activated during stimulus-induced transit to the membrane, particularly in endosomes(Salindas et al., 2004). Our study demonstrated that inhibition of iPLA₂ activity resulted in the retention of Src and pSrc/Tyr⁴¹⁶ in the ERC, which prevents Src from being

activated and that inhibition of Src activation had significant negative impact on the regulation of focal adhesion and ruffle formation, leading to inefficient chemotaxis.

In recent years, PLA₂s have been increasingly associated with various intracellular trafficking events, such as the tubule formation from the Golgi complex and endosomes, and membrane fusion events in the secretory and endocytic pathways(37). It has been generally accepted that hydrolysis of glycerophospholipids by phospholipase can cause the formation of lysophospholipids, leading to the formation of membrane curvature or tubules(38, 39). Insertion of lysophospholipids into artificial liposomes can result in the formation of membrane tubules(40). The generation of membrane-associated lysophospholipids by iPLA₂ has been suggested to be involved in the formation of Golgi membrane tubules in response to BFA and these tubules have been suggested to function in various trafficking pathways(41). Similarly, inhibition of lysophospholipid reacylation by pharmacological inhibitors of a Golgi-complex-associated lysophospholipid acyltransferase (LPAT) activity induces the rapid tubulation of Golgi membranes(42, 43). Various PLA₂ antagonists caused a block in the endocytic recycling pathway of Tf or TfRc, which is consistent with our results(44). Our study demonstrated that c-Src was retained at the ERC in the perinuclear area of iPLA₂β-KD cells as well as in cells treated with BEL, and that the Src trafficking defect can be rescued by the addition of exogenous AA. Addition of AA to BEL-treated cells and iPLA₂β-KD cells restores cSrc trafficking via the formation of small vesicles or tubules with which c-Src is associated. This suggests that AA, rather than lysophospholipid, plays a critical role in Src trafficking.

Our study demonstrated that cSrc-GFP is retained in the Rab7- and Rab11-positive compartments in BEL-treated or iPLA₂β-KD cells, and that AA can restore the formation of Rab11-positive recycling endosomes. These results indicate that AA is specifically required for the formation/trafficking of Rab11-positive recycling endosomes. Perinuclear localization of Src, which colocalizes with endosomal markers and the TGN, has been demonstrated in a previous study(24). Early studies have shown the function of Rab11 in endocytic recycling from the endosomal recycling compartment (ERC) to the plasma membrane(45, 46). Our results are consistent with the earlier finding that the expression of dominant negative Rab11 results in c-Src retention in the perinuclear region(24). There might be at least three processes that require AA during vesicle formation/trafficking. First, the formation of vesicles or tubules can be driven by the change of the lipid microenvironment, which requires product(s) of phospholipase. Since rafts primarily contain saturated fatty acids in a highly ordered manner, the saturated fatty acids attached to proteins would drive insertion of these modified proteins into rafts(47). Early study showed that recycling endosomes are highly enriched with lipids and proteins generally believed to associate into membrane microdomains(48). Thus, the lipid microenvironment within recycling endosomes is likely unique and require tight regulation. AA might be required for inducing changes in the lipid architecture of the ERC membrane, allowing the formation/release of RE. There was an interesting report that induction of Src activity in rafts was slower and weaker whereas the induction of Src activity in nonrafts was faster and stronger(49). Therefore, AA might have a direct impact on the induction of Src activation. Second, AA might be required for the interaction between kinesin and cargo membrane. Kinesin motor proteins have been shown to facilitate vesicle formation/trafficking by pulling membrane tubules or vesicles along on microtubules. Some of these kinesins have a tail domain that can interact with phospholipids. Unc104/KIF1A has a PH domain located at the C-terminal tail, which directly interacts with PI-(4,5)-bisphosphate (PIP₂) and transports PIP₂-containing vesicles(50, 51). The PX motif of KIF16B binds PI(3)P and localizes to membranes bearing this lipid *in vivo*(52). Tracking of cSrc-GFP vesicles upon AA addition in iPLA₂β-KD cells clearly indicate the increase of directionality and speed of vesicles. AA might play an important role in the regulation of interactions between the tail domain of

kinesins and cargo vesicles. Third, AA might affect lipid modification of Src. It has been suggested that lipid modification plays an important role in spatial activation and membrane delivery of Src family kinases. Dynamic exchange of c-Src between the plasma membrane and endosomes has been reported while palmitoylated Lyn was exocytosed to the membrane through the Golgi (53). Localization of Fyn to lipid rafts has been shown to be affected by the lipid modification as modification with unsaturated or polyunsaturated fatty acids reduced its raft localization(54). Src modified with unsaturated fatty acids would be excluded from rafts since it would be energetically unfavorable to pack unsaturated fatty acids into the highly ordered raft domain. Our results that BODIPY-AA retains biological function in inducing Akt phosphorylation practically rules out this possibility since BODIPY-AA can not be used for acylation. However, it is still possible that AA might affect acylation of Src since longer chain fatty acyl CoAs, including stearate and arachidonate, have been reported to compete with palmitate for incorporation into Fyn(55).

In summary, we demonstrated a critical role of AA in the regulation of microglia chemotaxis. AA is required for the cSrc trafficking to the plasma membrane via controlling the release/formation of recycling endosomes from the ERC.

Materials and Methods

Cell culture and Transfection

BV2 microglia cells were maintained in Dulbecco's modified Eagle's medium (DMEM) MEM supplemented with 10% FBS and penicillin-streptomycin (Gibco BRL, Grand Island, NY). We have used lentivirus-mediated short hairpin RNA (shRNA) for gene knockdown. MISSION shRNA clones from Sigma (NM_016915.2-2305s1c1 for iPLA₂β; NM_007783.2-1317s1c1 for c-Src; NM_008054.1-1451s1c1 for Fyn) containing hairpin sequences were used for iPLA₂, c-Src, and Fyn knockdown. Lentivirus-infected cells were selected for stable cell lines in the presence of puromycin. For rescue experiments, human iPLA₂-GFP that is RNAi-resistant was transfected into iPLA₂β-KD cells to examine chemotaxis and Src trafficking. Cells were transfected with Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions, with Cerulean-HDEL, VSV-G-YFP, pEGFP-cSrc, mCherry-Rab11 and mCherry-Rab7 and cultured for 12 h. All experiments were performed 32 h after transfection.

Chemotaxis Assay

Migration assays were performed as previously described (17, 56). Briefly, Transwell chamber membranes (6.5-mm diameter, 8 mM pore size; Corning, Corning, NY) were coated with 3 μg/ml of fibronectin for 8 h. For chemotaxis assay, either serum-free DMEM medium or serum-free DMEM medium containing 100 μM ADP was added to the lower chamber. Cells suspended in serum-free DMEM were added to the upper chamber. Pharmacological inhibitors, LY294002 (20 μM; Promega, Madison, WI); PP2 (10 μM; Alexis, San Diego, CA); BEL (5 μM; Sigma, St. Louis, MO), were added to the upper chamber. After incubating for 6 h, non-migrating cells were removed from the upper chamber with a cotton swab and cells that had migrated to the lower surface of the membrane were fixed with 3.7% formaldehyde for 10 min and stained with 0.2% crystal violet. Cells were imaged and intensity of staining was measured *image quant* software.

Measurement of free ³H-Arachidonic Acid

Cells were labeled with ³H-arachidonic acid (0.5 μCi per plate; 69.08 Ci/mM) for 6 hr in serum-free DMEM. Labeled cells were pretreated with or without 5 μM BEL for 30 min and then stimulated with 100 μM ADP for 5 min. Cells were resuspended in PBS and immediately centrifuged at 13,000 RPM for 1 min and supernatant was removed. Cell

pellets were sonicated on ice after addition of 100 μ l of PBS and 500 μ l of dichloromethane, followed by vortex mixing for 1 min. The samples were spun in a microcentrifuge for 3 min. The lower organic phase was transferred to a new tube, and the solvent was evaporated under a stream of nitrogen. The residue was dissolved in 100 μ l of methanol, and a 20 μ l aliquot was counted for 5 min using a Packard Tri-carb scintillation counter.

Immunofluorescence stainings

Control microglia (BV2) and iPLA $_2$ β -KD cells were attached to glass coverslips coated with 3 μ g/ml of fibronectin. Following 12-24 h cultures, when cells were 60-80% confluent, cells were washed and starved for 4 h in serum-free DMEM. Cells were pretreated with pharmacological inhibitors and stimulated with 100 μ M ADP. Cells were washed once in PBS, fixed in 3.7% formaldehyde for 10 min in a 37°C incubator, permeabilized in 0.2% Triton X-100 for 10 min at room temperature, washed in PBS, and then blocked in 1% bovine serum albumin (BSA) in PBS for 20 min at room temperature. Primary Abs were diluted in 1% BSA in PBS at the following concentrations: anti-Src (1:1000; Cell Signaling), anti-phospho-Src, clone 9A6, Alexa Fluor 555 conjugate (1:1000; Upstate), anti-GM130 (1:2000; BD Biosciences), anti-CD-MPR (1:2000; Santa Cruz) and wheat germ agglutinin (WGA) Alexa Fluor 594 conjugate (1:5000; Molecular Probes). After 1 h of incubation, cells were washed in PBS and incubated with FITC-conjugated anti-rabbit antibodies or Texas-Red conjugated anti-mouse antibodies (Santa Cruz) in PBS for 1 h. Images were captured with Roper Cascade 1K digital camera and processed using the program Metamorph (Universal Imaging, Media, PA). For colocalization studies, cells were incubated in serum-free DMEM containing Alexa Fluor 568-conjugated transferrin (Invitrogen) or 100 nM LysoTracker Red DND-99 (Invitrogen) for 30 min at 37°C and washed twice with PBS and then medium was changed for live cell imaging. Live cell imaging was performed as described in the section Live fluorescence microscopy. Mouse monoclonal anti-Fyn and anti-iPLA $_2$ antibodies were from Santa Cruz.

Live Cell Fluorescence Microscopy

Transfected cells were attached to 35-mm glass-bottom dishes coated with 3 mg/ml of fibronectin. When cells reach 60-80% confluency, cells were starved for 4 hr in serum-free DMEM. BODYPY-AA, Alexa⁵⁹⁵-transferrin, or LysoTracker were added to the medium. Labeling with transferrin and LysoTracker performed as described above. Dishes were transferred to a heated (37°C) chamber and imaged fluorescence microscope with a 60X oil-immersion objective. Cells were monitored over a 30 min period by capturing images every 30 s. Acquisition was performed using Metamorph.

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Figure 1. iPLA₂ activity is required for ADP-induced Akt phosphorylation and chemotaxis in microglia

(A) Cells were pretreated with 20 μ M LY294002 for 20 min or 5 μ M BEL for 30 min, and then stimulated with 100 μ M ADP for 5 min or 30 μ M arachidonic acid (AA) for 30 min. Cells were lysed and subjected to western blot with rabbit anti-pAkt/Thr³⁰⁸ antibody. Quantification of Akt phosphorylation from five independent experiments is shown in the graphs. **, $p < 0.005$, Student's *t*-test. (B) Cells were assayed for chemotaxis toward 100 μ M ADP in the presence of various inhibitors using a transwell chamber assay. Cells that migrated to the bottom of the transwell membrane were stained with crystal violet, and were quantified using *image quant* software. Quantification of from five independent assays is shown in the graphs. *, $p < 0.01$; **, $p < 0.005$. (C) Activation of iPLA₂ activity upon ADP stimulation. Cells were labeled with ³H-AA for 6 hours and stimulated with ADP in the presence or absence of BEL. Free AA level was measured after MeOH/DCM extraction. (D) ADP-induced Akt phosphorylation was not affected by COX or LOX inhibitors. Cells were pretreated with 10 μ M Indomethacin, 10 μ M NS-398 (NS) for 30 min or 10 μ M Nordihydroguaiaretic acid (ND), 1 μ M Baicalein for 20 min. Cells were then stimulated with ADP and cell lysates were analyzed by western blotting using anti-pAkt/Thr³⁰⁸ antibody. (E) Effect of fatty acids on Akt phosphorylation. Cells were stimulated with 30 μ M Fatty acids – Arachidonic acid (AA), Oleic Acid (OA), Eicosapentaenoic acid (EPA), Dihomo- γ -linolenic acid (DGLA), or Linoleic acid (LA) – for 30 min and lysates were analyzed by western blotting using anti-pAkt/Thr³⁰⁸ antibody. *, $p < 0.01$ vs. Cont. (F) iPLA₂ activity is required for ADP-induced Akt and Src phosphorylation. iPLA₂-KD cells were stimulated with 100 μ M ADP for 5 min or 30 μ M AA for 30 min. Akt/Thr³⁰⁸ and Src/Tyr⁴²³ phosphorylation was examined by western blot. Quantification of Akt and Src phosphorylation from five independent experiments is shown in the graphs. Error bars indicate $\pm$ SEM. #, $p < 0.05$ vs. iPLA₂-KD Cont; *, $p < 0.01$ vs. BV2 Cont. Inset shows a western blot of iPLA₂ in control and iPLA₂-KD cells.

Figure 2. iPLA₂ regulates PI3K activation via Src activation

(A) Cells were pretreated with 5 μM BEL for 30 min or 10 μM PP2 for 20 min and then stimulated with ADP for 5 min. Cells were lysed and subjected to western blot with anti-pAkt/Thr³⁰⁸ antibody. Akt phosphorylation can be blocked by either BEL or PP2. Quantification of five western blots is shown in the graph. #, $p < 0.05$ vs. ADP alone. (B) Knock-down of cSrc, but not Fyn, blocks Akt phosphorylation upon ADP stimulation. Cell lysates were collected and analyzed by western blotting using anti-cSrc and anti-Fyn and anti-pAkt Thr³⁰⁸ antibodies. (C) Inhibition of iPLA₂, iPLA₂ knock-down, or inhibition of Src significantly reduced ADP-induced chemotaxis of microglia cells. Cells were assayed for chemotaxis toward 100 μM ADP in the presence of inhibitors using a transwell chamber chemotaxis assay. *, $p < 0.01$ vs. ADP alone. For rescue experiment, human iPLA₂β-GFP that is RNAi-resistant was transfected into iPLA₂β-KD cells. #, $p < 0.005$ vs. GFP control. (D) Src activity is required for the PI3K activation. BV2 cells were pretreated with 20 μM LY for 20 min and stimulated with 100 μM ADP. Cell lysates were collected after 2 or 5 min and analyzed by western blotting using anti-pAkt Thr³⁰⁸ and anti-pSrc/Tyr⁴²³ and / Tyr⁵³⁴ antibodies. Quantification of pAkt/Thr³⁰⁸ and pSrc/Tyr⁴²³ band intensity from four blots is shown in the graphs. Akt phosphorylation, but not Src, was abrogated by LY294002. #, $p < 0.05$. (E) iPLA₂, but not PI3K, activity is required for Src activation. Cells were pretreated with 20 μM LY for 20 min or 5 μM BEL for 30 min, and then stimulated with 100 μM ADP for 5 min or 30 μM AA for 30 min. Cells were lysed and subjected to western blot with anti-pSrc/Tyr⁴²³ antibody. Error bars indicate $\pm$ SEM. #, $p < 0.05$; *, $p < 0.01$.

Figure 3. iPLA2 activity is required for focal adhesion and ruffle formation

(A) Changes of focal adhesion size and number were assessed by paxillin immunostaining. Cells were plated on coverslips coated with 3 μ g/ml fibronectin. After pretreatment with BEL, cells were stimulated with 100 μ M ADP for 5 min. Immunostaining was performed on fixed cells with anti-mouse paxillin antibody. (B) Kymography analysis of membrane ruffles formation. Cells were pretreated with BEL and then stimulated with 100 μ M ADP. Lamella dynamics was then analyzed by kymographs.

Figure 4. Inhibition of iPLA₂ activity significantly impaired Src/pSrc localization to the plasma membrane

(A) Microglia cells were pretreated with or without 5 μ M BEL for 30 min, and then stimulated with 100 μ M ADP for 5 min or 30 μ M AA for 30 min. Immunostaining was performed on fixed cells with rabbit anti-Src and mouse-pSrc/Tyr⁴²³ antibodies. Src translocation to the plasma membrane was impaired in BEL-treated cells and addition of AA appears to restore Src translocation upon ADP stimulation. Bar = 10 μ m. (B) Fluorescent intensity of Src and pSrc along the plasma membrane, quantified from the images, are shown in the graphs. *, $p < 0.001$ vs. Cont; **, $p < 0.001$ vs. ADP alone; ***, $p < 0.001$ vs. BEL+ADP. (C) AA addition or expression of human iPLA₂ β -GFP could restore Src trafficking to the plasma membrane in iPLA₂-KD cells. iPLA₂-KD cells were stimulated with or without 30 μ M AA for 30 min. Human iPLA₂ β -GFP was transfected into iPLA₂ β -KD cells to examine the rescue of Src trafficking. Immunostaining was performed on fixed cells with rabbit anti-Src and mouse-pSrc/Tyr⁴²³ antibodies. (D) Motility of cSrc-GFP vesicles in iPLA₂-KD cells. Cells were transfected with cSrc-GFP construct and vesicles associated with cSrc-GFP were tracked before and after AA addition. Trajectories of vesicle movement for 30 min are shown. Directional movement of cSrc-GFP vesicles toward the plasma membrane was observed after AA addition.

Figure 5. Inhibition of iPLA₂ activity causes Src to be remained at ERC

(A) Microglia cells were pretreated with 5 μ M BEL for 30 min, and then cells were fixed, permeabilized, and stained with anti-Src and various organelle markers (anti-GM130, Alexa⁵⁹⁵-WGA, or anti-CD-MPR). Cells transfected with Cerulean-HDEL were fixed 32 hr after transfection and stained with anti-Src antibody. GFP-cSrc and Cherry-Rab11 or GFP-cSrc and Cherry-Rab7 transiently coexpressed in iPLA₂-KD cells were imaged 32 hr after transfection in living cells. The region outlined by the box is shown at higher magnification on the bottom panels. Bar = 10 μ m. The percent colocalization of Src or GFP-cSrc that colocalized with organelle markers or endosomal markers is shown. (B) iPLA₂-KD and control cells were incubated with AlexaFluor⁵⁶⁸-transferrin for 30 min at 37°C. Cells were washed with PBS and live cell images were taken at 0 min or 30 min after wash. Quantification of transferrin fluorescent intensity in the ERC areas is shown in the graph. The graph represents two independent experiments in which 10 cells were analyzed for each condition. *, $p < 0.001$ vs. Control 30 min. (C) iPLA₂-KD cells were transiently transfected with GFP-cSrc construct and then incubated with AlexaFluor⁵⁶⁸-transferrin for 30 min at 37°C and washed with PBS. Live cell images were taken 30 min after wash.

Figure 6. AA is required for c-Src delivery to PM from ERC

(A) iPLA₂-KD cells were co-transfected with cSrc and Cherry-Rab7 or Cherry-Rab11 constructs and then cells were stimulated with 30 μ M AA. Live cell images were taken every 30 sec for 30 min. Colocalization of GFP-cSrc and Cherry-Rab7 or Cherry-Rab11 was examined in the merged image. (B) VSV-G trafficking to the membrane was not affected by BEL. Microglia cells were transfected with VSV-G-YFP construct and kept at 40°C or shifted to the permissive temperature 33°C for 30 min in the absence or presence of 5 μ M BEL. Cells were then fixed and stained with anti-Src-antibody. Fluorescent intensity of Src and VSV-G along the plasma membrane, quantified from the images, is shown in the graph. *, $p < 0.01$ vs. 40°C Cont Src; **, $p < 0.005$ vs. 40°C Cont VSV-G-YFP. (C) H-Ras trafficking is not affected in iPLA₂-KD cells. Cells were transfected with GFP-H-Ras and Cherry-Rab11 constructs and live cells images were taken.

Figure 7. Enrichment of BODIPY-AA in recycling endosomes

(A) Activation of PI3K signaling by BODIPY-AA. AA or BODIPY-AA was incubated with iPLA₂-KD cells for 30 min and phosphorylation of Akt at Thr³⁰⁸ was examined by western blotting. Quantification of pAkt/Thr³⁰⁸ band intensity from five blots is shown in the graphs. *, $p < 0.05$ vs. Cont. (B) Formation of recycling endosomes associated with pSrc/Tyr⁴²³ by the addition of BODIPY-AA to iPLA₂-KD cells. AA was added to iPLA₂-KD cells and cells were fixed and stained with pSrc/Tyr⁴²³ antibody. (C) Selective enrichment of BODIPY-AA in recycling endosomes.

Mixture of AA and BODIPY-AA (10:1, 20 μ M total) was added to iPLA₂-KD cells transfected with mCherry-Rab7 or – Rab11, or cells were labeled with Lysotracker 30 min after addition of AA. Colocalization of BODIPY-AA with Rab11, but not with Rab7 or Lysotracker, was observed. The percent colocalization of BD-AA with lysotracker, Rab7, or Rab11 was quantified and shown. Bar = 20 μ m.