

Signal-activated phospholipase regulation of leukocyte chemotaxis

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Abstract Signal-activated phospholipases are a recent focus of the rapidly growing field of lipid signaling. The extent of their impact on the pathways regulating diverse cell functions is beginning to be appreciated. A critical step in inflammation is the attraction of leukocytes to injured or diseased tissue. Chemotaxis of leukocytes, a requisite process for monocyte and neutrophil extravasation from the blood into tissues, is a critical step for initiating and maintaining inflammation in both acute and chronic settings. Recent studies have identified new important and required roles for two signal-activated phospholipases A₂ (PLA₂) in regulating chemotaxis. The two intracellular phospholipases, cPLA₂α (Group IVA) and iPLA₂β (Group VIA), act in parallel to provide distinct lipid mediators at different intracellular sites that are both required for leukocytes to migrate toward the chemokine monocyte chemoattractant protein-1. This review will summarize the separate roles of these phospholipases as well as what is currently known about the influence of two other classes of intracellular signal-activated phospholipases, phospholipase C and phospholipase D, in regulating chemotaxis in eukaryotic cells, but particularly in human monocytes. The contributions of these phospholipases to chemotaxis both *in vitro* and *in vivo* will be highlighted.—Cathcart, M. K. Signal-activated phospholipase regulation of leukocyte chemotaxis. *J. Lipid Res.* 2009. 50: S231–S236.

Supplementary key words chronic inflammation • macrophage • lipid mediators • monocyte

Studies exploring the mechanisms regulating cell movement have often focused on protein receptors, protein kinase cascades, and protein-protein interactions, paying less attention to the importance of lipids in controlling migration. The exception to this has been the broad acceptance of the importance of phosphatidyl inositol phosphates and their kinases/phosphatases in regulating cell migration. A

relatively recent focus of studies characterizing the roles of lipids in signaling has been on the role of signal-activated phospholipases, enzymes that metabolize glycerophospholipids to generate bioactive products capable of regulating downstream effector pathways. These studies have made us realize how little we understand about the importance of lipid mediators in regulating cell function.

Primary blood leukocytes are one of the most commonly employed cell types used for investigating chemotaxis of human cells. Leukocytes are white blood cells that are major participants in inflammatory responses and are central mediators of inflammation, with neutrophils and monocyte/macrophages serving as the prime effector cells of acute and chronic inflammation, respectively. In response to an inflammatory stimulus, blood leukocytes adhere to and cross the endothelium of the blood vessel wall and then home to various tissue sites or participate in inflammatory reactions in the vascular wall, depending on the location and nature of the particular tissue injury or inflammatory stimulus. The migration of leukocytes from the blood into tissue is driven by chemotactic factors called chemokines, a group of small molecular weight cytokines that function as cell attractants in a gradient-dependent fashion. These potent molecules are ligands for G-protein-coupled transmembrane receptors.

Much of what we know about signal transduction pathways that regulate eukaryotic cell chemotaxis has been determined from studies in the *Dictyostelium* slime mold model system. Some signaling pathways shown to regulate chemotaxis are shared between *Dictyostelium* and leukocytes, while others are not. In most cases, pathways have first been identified to play a role in *Dictyostelium* and then further explored in leukocytes for their relevance. An

Abbreviations: AA, arachidonic acid; Cox, cyclooxygenase; cPLA₂, cytosolic phospholipase A₂; Cyp, cytochrome p450 epoxygenase; EET, epoxyeicosatrienoic acids; iPLA₂, calcium-independent phospholipase A₂; LO, lipoxygenase; LPA, lysophosphatidic acid; MCP-1, monocyte chemoattractant protein-1; PA, phosphatidic acid; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D; PPARγ, peroxisome proliferator-activated receptor γ.

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exception to this is the recently identified role that phospholipases A₂ (PLA₂) have in regulating the speed of migration and the directionality of leukocyte chemotaxis. Both cytosolic phospholipase A₂ (cPLA₂) and calcium-independent phospholipase A₂ (iPLA₂), the intracellular signal-activated PLA₂, were first discovered to regulate chemotaxis in leukocytes (1, 2). The homolog of iPLA₂ was later shown to regulate chemotaxis in *Dictyostelium*, whereas cPLA₂ does not seem to have a counterpart in this organism (3, 4). Recent studies in *Dictyostelium* have revealed a complex set of parallel pathways that play lesser or greater roles in regulating chemotaxis depending on the steepness of the chemokine gradient and the developmental stage of this organism [(3, 4) and reviews (5, 6)]. Therefore, it is critical to examine the relevance of particular pathways anew in leukocytes prior to presuming their participation in the chemotactic response.

Monocyte chemoattractant protein-1 (MCP-1) (also known as CCL2) is an example of a chemotactic factor that is important in both acute and chronic inflammatory responses. *In vivo* studies have revealed that it is predominantly a chemokine for attracting monocytes. Despite *in vitro* reports of other chemokines with the capacity to attract monocytes, studies in MCP-1-deficient mice have revealed that MCP-1 is solely responsible for monocyte recruitment in a variety of inflammatory settings (7). Furthermore, MCP-1 expression has been documented in disorders characterized by mononuclear cell infiltrates, suggesting that it contributes to the inflammatory component of such diseases as atherosclerosis, allergy, multiple sclerosis, or rheumatoid arthritis (8, 9). Although MCP-1 is believed to be important in these disease processes, until recently, relatively little was known about how MCP-1 signals through its receptor, CC chemokine receptor 2, to induce monocyte chemotaxis. This defined chemokine-induced chemotactic response for primary human monocytes serves as a relevant model system for identifying lipid regulatory pathways, assessing their relevance *in vivo* and designing future therapies for targeting these novel pathways for anti-inflammatory treatments.

This review will summarize our current understanding of how signal-activated phospholipases and their products regulate eukaryotic cell migration in general and specifically focus on chemotaxis of primary human monocytes to MCP-1. The *in vitro* versus *in vivo* roles for these signal-activated phospholipases will be discussed as well as their potential to serve as targets for controlling inflammatory responses.

PLA₂

PLA₂ enzymes hydrolyze the fatty acyl group from the *sn*-2 position of glycerophospholipids resulting in the generation of free fatty acid [e.g., arachidonic acid (AA)] and lysophospholipid (e.g., lysophosphatidyl choline). Leukocytes have three broad classes of PLA₂: 1) secretory, referred to as sPLA₂ (Groups II and X); 2) calcium-dependent cPLA₂ (Group IV); and 3) iPLA₂ (Group VI). Only two of these

are intracellular signal-transducing, signal-activated phospholipases, cPLA₂ and iPLA₂. In this section, the focus will be on the two intracellular, signal-activated PLA₂, cPLA₂ and iPLA₂, and specifically cPLA₂α and iPLA₂β. Activity of these phospholipases can be inhibited by a variety of pharmacologic inhibitors, and antisense oligodeoxynucleotides have been designed that specifically block the expression of cPLA₂ and iPLA₂ (1, 10).

cPLA₂ regulation of chemotaxis

cPLA₂ is an intracellular, 110 kDa enzyme that requires nanomolar to micromolar concentrations of calcium to become activated. It is the only identified PLA₂ that selectively cleaves *sn*-2 AA and therefore significantly contributes to agonist-induced AA release and the formation of potent AA metabolites (11). cPLA₂ activity is induced by protein phosphorylation and calcium-dependent translocation to membranes from the cytosol (12). Protein kinase C has been shown to regulate cPLA₂ phosphorylation and activity in activated human monocytes (13–15).

An important role for cPLA₂ in regulating monocyte chemotaxis was first reported by Locati et al. (2) and later confirmed by our group (1). The importance of AA liberated by this enzyme was documented in this latter report. The distinctive translocation of this enzyme and the importance of cPLA₂ in regulating the speed of chemotaxis but not gradient sensing and directionality were reported in more recent studies (1, 16). Inhibition of cPLA₂ expression or pharmacologic inhibition of cPLA₂ enzymatic activity significantly impaired monocyte chemotaxis. Normal chemotactic activity, in cPLA₂-deficient monocytes, was restored by treatment with AA, one of the predicted products of this enzyme. Furthermore, cPLA₂ was found to translocate from the cytosol to the endoplasmic reticulum upon exposure of leukocytes to the chemotactic factor MCP-1.

AA has been speculated to regulate monocyte chemotaxis by being converted to bioactive eicosanoids via cyclooxygenase (Cox), lipoxygenase (LO), or cytochrome p450 epoxygenase (Cyp) enzymes. Inhibitors of Cox1/2 and 5-LO were used to determine whether these enzymes were essential for regulating monocyte chemotaxis to MCP-1. The data suggest that Cox1, Cox2 (both inhibited by indomethacin), and 5-LO (inhibited by MK886) are not required pathways for monocytes to chemotactically respond to MCP-1 since neither of these drugs affected chemotaxis (unpublished observations). Therefore, the current focus of these studies is on the potential contributions of the products formed by the cytochrome p450 enzyme pathway. Important roles for Cyp products and soluble epoxide hydrolase in inflammatory processes have been defined through the use of several urea-based inhibitors (17). This general role in inflammation may be related to the role that Cyp products are playing in leukocyte chemotaxis.

Little is known about the Cyp enzymes or their products and cell migration. This is primarily due to the recently identified fact that Cyp enzymes are remarkably labile in cell culture and most cell lines have lost expression of this enzyme family. As a result, this pathway merits a closer look

for its role in regulating chemotaxis since the technical limitations of prior studies resulted in underestimating the potential impact of the epoxyeicosatetraenoic acid (EET) products of these enzymes in biologic responses and since EETs have been shown to promote cell migration in certain systems (18). AA and EETs have been reported to cause increases in intracellular calcium through both store-operated calcium channels and calcium influx (19–23). The location of cPLA₂ in the endoplasmic reticulum is consistent with a possible role of Cyp products in regulating localized calcium release from intracellular stores. Calcium increases could certainly affect events critical for monocyte migration.

It is also possible that AA itself, and not a metabolite, is the regulator of cPLA₂-dependent chemotaxis, and based on prior work, one cannot ignore the fact that AA may regulate chemotaxis by modulating the production of reactive oxygen species, since cPLA₂-generated AA is essential in regulating the production of O₂⁻ by activated human monocytes via NADPH oxidase as well as regulating intracellular calcium levels (10, 14, 15). Prior results obtained in the *Dictyostelium* model have suggested that AA can indeed enhance chemotaxis by affecting calcium influx, and it was proposed that increased intracellular calcium might in turn regulate iPLA₂ activity [see review (24)]. Another potential regulatory function of cPLA₂-derived AA is in binding to proteins and facilitating their association with the plasma membrane (e.g., a regulatory kinase or a chaperone for a regulatory enzyme similar to the S100A8/A9 function in facilitating NADPH oxidase complex formation and activity) (25).

iPLA₂

iPLA₂ is an intracellular, 85 kDa protein that requires no calcium for its catalytic activity. iPLA₂ has no acyl specificity but prefers phosphatidic acid (PA) as a substrate. iPLA₂ has been suggested to function in the steady-state remodeling of phospholipid fatty acyl groups (26); however, recent data implicate this enzyme as an active participant in signal transduction pathways regulating chemotaxis (1, 3, 4, 16). Little is known about the regulation of iPLA₂ activity in any cell type.

Studies using pharmacologic inhibitors and specific *in vitro* knockout with antisense oligodeoxyribonucleotides demonstrated a critical role for iPLA₂β in monocyte chemotaxis to MCP-1. When exposed to MCP-1, iPLA₂ translocated from the cytosol to the plasma membrane, and upon cell polarization, iPLA₂ became concentrated in the pseudopod at the forward leading edge of the cell toward the highest concentration of the chemokine. iPLA₂ colocalized with both membrane markers and CDC42, a Rho family member. Rho proteins are important regulators of actin organization and focal complex formation (1, 16).

In contrast with cPLA₂, the lipid product that restored chemotaxis in cells rendered deficient in iPLA₂ was found to be lysophosphatidic acid (LPA) and not AA. To understand how iPLA₂ and iPLA₂-derived LPA regulated chemotaxis, detailed analyses of monocyte chemotaxis were performed. The number of migrating cells, their speed,

and their directionality of migration were analyzed in monocytes with and without iPLA₂ expression. Both speed and directionality were significantly influenced by iPLA₂, whereas mostly speed of migration was affected by cPLA₂ (16).

The influence of iPLA₂ on directionality can be likened to that of a compass pointing toward the highest concentration of the chemokine. The determination of directionality must involve regulation of the cytoskeleton for controlling the direction of forward membrane protrusion and cell orientation. Exposure of monocytes to MCP-1 induces profound morphologic changes noted by greater adhesion and cell spreading. These correlate with an increase in F-actin polymerization. All of these changes were blocked in iPLA₂-deficient monocytes and remarkably restored by addition of LPA (16).

Although long regarded as a metabolic intermediate, LPA has recently been the focus of numerous studies identifying it as a multifunctional signaling molecule. LPA, the simplest glycerophospholipid, is more hydrophilic than other members, and unlike other lysophospholipids, it is not lytic due to its small headgroup [reviewed in (27)]. LPA may serve to regulate monocyte chemotaxis by its intracellular generation and action or by leaving the monocyte and interacting with one of the previously characterized cell surface LPA receptors. To date, the only intracellular receptor identified for LPA is peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor (28). Since regulation of chemotaxis by iPLA₂ and LPA is observed in a chemotaxis assay of 90 min duration, PPARγ is not a likely target because it would have limited time to alter gene expression. Because LPA is presumably generated in the leading edge of the monocyte, where iPLA₂ translocates and becomes concentrated, it is quite possible that it has a direct function in that location. Alternatively, LPA may leave the cell and interact with one of the previously characterized LPA receptors. There are currently five receptors that have been identified as LPA receptors, LPA_{1–5} (29). Among these, monocytes are reported to express only LPA₁ and LPA₂ (30, 31). The potential roles of PPARγ and monocyte LPA receptors in allowing LPA to rescue the chemotactic response of iPLA₂-deficient or iPLA₂ inhibitor-treated monocytes need to be explored.

To summarize this section, signal-activated cPLA₂ and iPLA₂ are both required for monocyte chemotaxis to MCP-1. These enzymes are both activated by MCP-1, translocate to distinct intracellular sites, and provide different and requisite lipid products that control monocyte chemotaxis (Fig. 1). The chemotaxis of iPLA₂-deficient cells that could no longer respond to MCP-1 were rescued by treatment with LPA but not by AA. LPA regulates actin polymerization in the pseudopod, and iPLA₂ activity is important for determining monocyte directional migration and speed of migration. In contrast with iPLA₂, the chemotaxis of cPLA₂-deficient cells was rescued by treatment with AA and not by treatment with LPA. cPLA₂ was shown to regulate the speed of monocyte migration in response to MCP-1 from its location in the endoplasmic reticulum. It is important to stress that treatment of monocytes with either AA or LPA alone

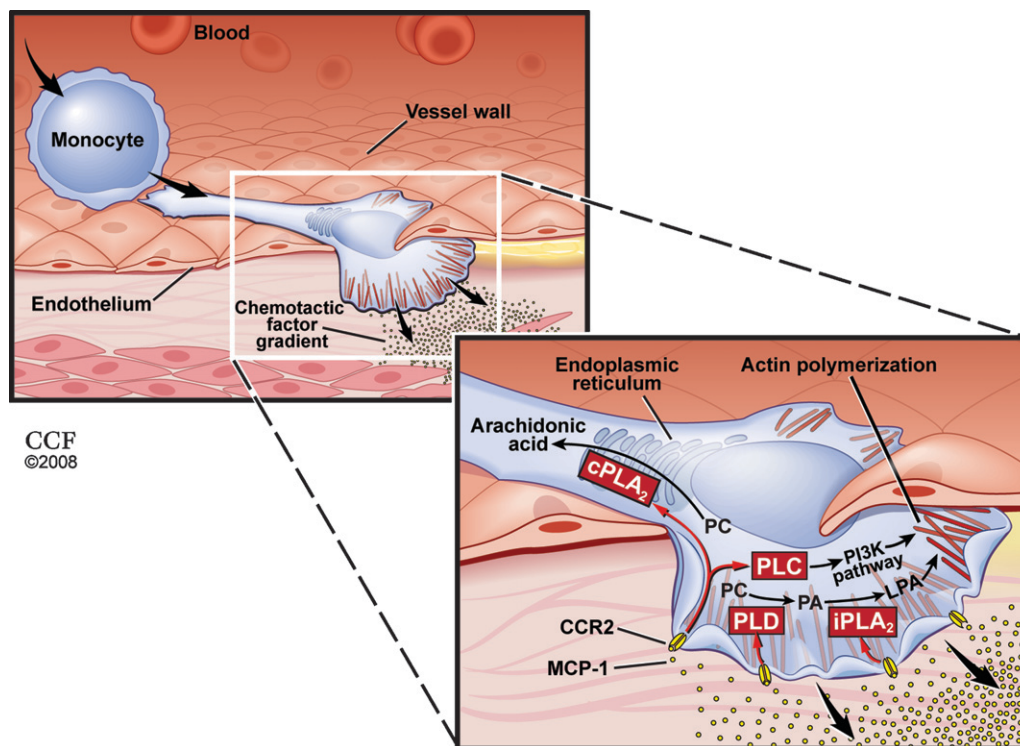


Fig. 1. Circulating blood monocytes adhere to activated endothelium at sites of disease or injury. The monocyte becomes polarized and forms a pseudopod in response to a chemical gradient of chemotactic factor production like MCP-1 and follows the increasing concentration of the chemotactic factor into the tissue. PLA₂ translocate to distinct compartments: iPLA₂, PLC, and PLD to the pseudopod at the leading edge and cPLA₂ to the endoplasmic reticulum posterior to the nucleus. LPA is generated by iPLA₂ at the leading edge of the cell where it regulates actin polymerization and protrusion of the pseudopod. LPA also facilitates detection of the MCP-1 gradient since it was shown to regulate not only the speed but also the directionality of monocyte migration. AA is generated by cPLA₂ and regulates the efficiency of monocyte migration through as yet unknown mechanisms. One regulatory role of PLD is its apparent upstream regulation of PA production that serves as a substrate for iPLA₂ generation of LPA. PLC can influence chemotaxis by regulating actin polymerization via the phosphatidyl inositol 3-kinase pathway.

does not induce monocyte migration or enhance MCP-1-mediated chemotaxis under these conditions (1). These lipids appear to be overcoming the specific blocks in cPLA₂ and iPLA₂ activity and expression, respectively.

PHOSPHOLIPASES C AND D

Phospholipase C (PLC) hydrolyzes phospholipids [e.g., phosphatidyl-inositol-(4,5)-biphosphate]. So far, 13 PLC isoenzymes have been identified belonging to six different groups (32). This activity generates cytosolic inositol phosphates, such as inositol-(1,4,5)-triphosphate and diacylglycerol. Inositol phosphates stimulate the release of intracellular calcium, thereby influencing intracellular calcium levels and the activation of calcium-dependent enzymes (e.g. cPLA₂ and protein kinase C). Diacylglycerol also serves as a substrate for diacylglycerol kinases and is a potent activator of certain protein kinase C isoforms. Each of these pathways can regulate the processes of cell motility.

Studies in *Dictyostelium* suggest that PLC regulates the phosphatidyl inositol 3-kinase-mediated chemotaxis pathway and F-actin polymerization at the leading edge

[reviewed in (24)]. In leukocytes, the role for PLC in regulating chemotaxis is rather confusing, with evidence supporting a role in some chemotactic factor-induced biologic responses, such as superoxide anion production and calcium signaling, while not influencing chemotaxis. In *Dictyostelium*, PLC has been reported to regulate the localization of the phosphatase and tensin homolog PTEN and actually inhibit chemotaxis to cyclic AMP (33). The role for PLC may ultimately prove to be both cell type and chemotactic factor dependent. The contributions of PLC to leukocyte chemotaxis deserve further study.

The action of phospholipase D (PLD) on its substrate phosphatidylcholine (or other glycerophospholipids) results in the formation of PA and choline (or other head group) as a result of an attack on the phosphodiester bond. Among the phospholipases, relatively little is understood about how PLD contributes to leukocyte chemotaxis. Studies on neutrophils responding to the formyl peptide (formylMetLeuPhe) suggest that PLD activity may regulate inside-out activation of the β 2 integrin CD11b/CD18 that is important for influencing leukocyte adhesion and migration (34, 35). This appears to be due to PA regulation of the generation of PtdIns(4,5)P₂ and promotion of talin

binding to the cytosolic domain of CD18. When PLD activity was blocked, PA restored migration. Other investigators did not see this same effect of PLD on formylMetLeuPhe-induced neutrophil chemotaxis, and the reasons for this discrepancy are not clear at this time but may be related to the very different chemotaxis assays employed in these two studies (36).

Recent studies in our lab indicate that MCP-1 induces rapid activation of PLD and that pharmacologic inhibition of PLD results in inhibition of PLD activity in concert with inhibition of monocyte chemotaxis to MCP-1. When PLD activity was inhibited, monocyte chemotaxis to MCP-1 was restored when PA was added to the monocytes (unpublished observations). Since PLD-derived PA can serve as a substrate for subsequent iPLA₂ cleavage generating LPA and free fatty acid, we surmise that PLD activity is an upstream requirement for providing PA as a substrate for iPLA₂, thereby regulating monocyte chemotaxis via the iPLA₂ pathway discussed earlier in this review (Fig. 1).

IN VIVO VALIDATION


It does not necessarily follow that a dependence of monocyte chemotaxis on a signal-activated phospholipase activity *in vitro* implies that this also happens *in vivo*. There are many reasons, not the least of which is the complexity of the *in vivo* system, that *in vitro* findings may prove invalid *in vivo*. It is therefore necessary to validate the significance of these pathways *in vivo* in inflammatory responses. For studies characterizing pathways that regulate MCP-1-dependent chemotaxis, it is fortuitous that thioglycollate-induced monocyte peritoneal inflammation in mice has been shown to be dependent on MCP-1. This model was used to test the relevance of both iPLA₂ and cPLA₂ for regulating inflammation *in vivo* by conducting adoptive transfer of monocytes rendered deficient in iPLA₂ or cPLA₂ (16). These studies indicated that both phospholipases are indeed critical for *in vivo* monocyte chemotaxis and thereby suggest that these pathways may serve as opportune targets for intervention in the process of inflammation. The adoptive transfer model system was performed using tagged mouse monocytes transferred to recipient animals. This model has also been used to monitor the trafficking of human monocytes in mice during peritonitis, and human monocytes were shown to traffic similarly to mouse monocytes (37). Therefore, this adoptive transfer model can be used to assess the relevance of a particular enzyme for regulating human monocyte chemotaxis. Similar studies are necessary for validating other pathways for *in vivo* relevance.

FUTURE THERAPEUTIC APPROACHES

Although the much of what we know about the roles for signal-activated phospholipases in human cells is derived from lipid regulation of monocyte and neutrophil chemotaxis, the relevance of these findings implicating critical roles for signal-activated phospholipases to cell migration in

general remains to be elucidated. Data suggest that signal-activated phospholipases contribute to chemotaxis of many cell types, including monocytes, vascular smooth muscle cells, endothelial cells, and neutrophils (unpublished observations). The requirement for signal-activated phospholipases and their lipid products in controlling leukocyte chemotaxis makes them a logical target for therapeutic intervention. The possibility that these enzymes may be involved in regulating general cell movement in a more global sense would limit the value of targeting these pathways for controlling leukocyte recruitment. In this case, it would be critical to target this intervention specifically to monocyte/macrophages and/or neutrophils.

CONCLUSIONS

Signal-activated phospholipases play critical roles in regulating the chemotactic response, particularly in leukocytes. The exact mechanisms that are controlled by these phospholipases remain to be fully elucidated and merit future study. The information gleaned from this research will advance our understanding of the mechanisms that control this important biological event and will suggest novel therapeutic approaches for treatment of inflammatory diseases. 

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