Interplay between phosphoinositide lipids and calcium signals at the leading edge of chemotaxing ameboid cells☆

Joseph J. Falke[*] and **Brian P. Ziemba**
Department of Chemistry and Biochemistry and Molecular Biophysics Program, University of Colorado, Boulder, CO 80309-0596, USA

Abstract

The chemotactic migration of eukaryotic ameboid cells up concentration gradients is among the most advanced forms of cellular behavior. Chemotaxis is controlled by a complex network of signaling proteins bound to specific lipids on the cytoplasmic surface of the plasma membrane at the front of the cell, or the leading edge. The central lipid players in this leading edge signaling pathway include the phosphoinositides $PI(4,5)P_2$ (PIP_2) and $PI(3,4,5)P_3$ (PIP_3), both of which play multiple roles. The products of $PI(4,5)P_2$ hydrolysis, diacylglycerol (DAG) and $Ins(1,4,5)P_3$ (IP_3), are also implicated as important players. Together, these leading edge phosphoinositides and their degradation products, in concert with a local Ca^{2+} signal, control the recruitment and activities of many peripheral membrane proteins that are crucial to the leading edge signaling network. The present critical review summarizes the current molecular understanding of chemotactic signaling at the leading edge, including newly discovered roles of phosphoinositide lipids and Ca^{2+}, while highlighting key questions for future research.

Keywords

Phosphatidylinositol lipid; PH domain; C2 domain; PKC; PI3K; PDK1

1. Introduction

Plasma membrane signaling events play a central role in the directed migration of eukaryotic ameboid cells up concentration gradients of specific attractant molecules. Leukocytes including macrophages and neutrophils possess one of the most highly developed chemotaxis pathways, enabling efficient cell migration toward infection or tissue damage (Jin, 2013; Kolaczkowska and Kubes, 2013; Parent and Weiner, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011; Hawkins et al., 2010; Collins and Meyer, 2009). Many autoimmune diseases yield hyperactivation of leukocyte chemotaxis, while certain infectious diseases interfere with leukocyte chemotaxis (Kolaczkowska and Kubes, 2013; Fahey et al., 2013). Thus, a molecular understanding of the chemotaxis signaling pathway could facilitate the development of useful therapies targeting inflammation and infection. More broadly,

[*]Corresponding author. Tel.: +1 303 492 3503., falke@colorado.edu (J.J. Falke).

many eukaryotic cells possess the ability to migrate or grow up gradients of attractant molecules using the ubiquitous elements of the widely conserved chemotaxis pathway. In addition, specific cell types possess specialized pathway elements that optimize performance for particular applications. Chemotactic cells in which the chemotaxis pathway has been investigated at the molecular level include leukocytes (Jin, 2013; Kolaczkowska and Kubes, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011; Hawkins et al., 2010; Collins and Meyer, 2009), fibroblasts (Wei et al., 2012), endothelial cells (Tsai and Meyer, 2012), neurons (Zamburlin et al., 2013; Henle et al., 2011; Waite and Eickholt, 2010), muscle cells (Trovati et al., 2013), mammary epithelial cells (Zhu and Nelson, 2013), cancer cells (Li et al., 2013; Vadas et al., 2011), and free-living amoeboid organisms such as *Dictyostelium* (Weiger and Parent, 2012; Cai and Devreotes, 2011).

2. Leading edge lipid and Ca^{2+} signals

The first major step in chemotaxis is the polarization of the cell, which generates an active, ruffling region of plasma membrane, termed the leading edge, at the front end of the cell that largely controls the direction of cell movement (Fig. 1) (Jin, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011; Hawkins et al., 2010; Swaney et al., 2010; Kolsch et al., 2008; Mortimer et al., 2008; von Philipsborn and Bastmeyer, 2007; Bourne and Weiner, 2002). In general, leading edge formation and maintenance requires the appearance of two localized second messenger signals: the signaling lipid phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) (Jin, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011), and the signaling ion Ca^{2+} (Collins and Meyer, 2009; Wei et al., 2012; Tsai and Meyer, 2012; Zamburlin et al., 2013; Henle et al., 2011; Tian et al., 2010; Wei et al., 2009; Evans and Falke, 2007). While the importance of $PI(3,4,5)P_3$ at the leading edge membrane has long been recognized, Ca^{2+} has emerged as an essential leading edge signal more recently. Contemporary leading edge models ignored Ca^{2+} until local Ca^{2+} signals at the leading edge were found to be essential for macrophage leading edge stability, dynamics and function (Evans and Falke, 2007), to play a role in directional control of migrating fibroblasts and endothelial cells (Wei et al., 2012; Tsai and Meyer, 2012; Wei et al., 2009), and to be involved in the guidance mechanism of neuronal growth (Zamburlin et al., 2013; Henle et al., 2011; Heckman and Plummer, 2013). Thus in recent years Ca^{2+} has joined $PI(3,4,5)P_3$ as an essential second messenger in leading edge signaling (Collins and Meyer, 2009; Wei et al., 2012; Tsai and Meyer, 2012; Zamburlin et al., 2013; Henle et al., 2011; Tian et al., 2010; Wei et al., 2009; Evans and Falke, 2007; Heckman and Plummer, 2013; Schafer et al., 2012), with the possible exception of cells that lack constant external Ca^{2+} levels such as *Dictyostelium* (Wessels et al., 2012; Traynor et al., 2000). Besides $PI(3,4,5)P_3$ and Ca^{2+}, another second messenger – the signaling lipid diacylglycerol (DAG) and likely its phosphorylation product phosphatidic acid – is indirectly implicated at the leading edge (Tsai and Meyer, 2012; Abramovici et al., 2009). Finally, the constitutive plasma membrane lipid phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) plays crucial roles in the leading edge $PI(3,4,5)P_3$, Ca^{2+}, and putative DAG signals (Jin, 2013; Evans and Falke, 2007; Thapa and Anderson, 2012; Corbalan-Garcia et al., 2007; Corbin et al., 2007; Evans et al., 2006; Luo et al., 2004).

$PI(3,4,5)P_3$ signals are triggered by attractant binding to G-protein coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs), which activate membrane-targeted enzyme phosphoinositide 3-kinase (PI3K) (Jin, 2013; Vadas et al., 2011). This lipid kinase phosphorylates the constitutive lipid $PI(4,5)P_2$, yielding the signaling lipid $PI(3,4,5)P_3$. Since the substrate $PI(4,5)P_2$ is only ~2 mole % of the total lipid in the cytoplasmic leaflet of the plasma membrane, the $PI(3,4,5)P_3$ product density never exceeds this average level and is often a relatively rare bilayer component even during a peak signal (Corbin et al., 2004). The localized nature of the leading edge $PI(3,4,5)P_3$ signal arises both from the localized recruitment and/or activation of PI3K in this region, and from the suppressed membrane targeting or activity of the lipid phosphatase PTEN (phosphatase and tensin homologue) that hydrolyzes $PI(3,4,5)P_3$ back to $PI(4,5)P_2$ (1,4,5). Outside the leading edge region, active PTEN is bound to the plasma membrane and destroys $PI(3,4,5)P_3$ molecules emerging from the leading edge via diffusion (Cai and Devreotes, 2011).

The leading edge Ca^{2+} signal has been linked to both influx through plasma membrane Ca^{2+} channels and efflux from internal Ca^{2+} stores, although certain cell types may employ only one of these mechanisms (Wei et al., 2012; Tsai and Meyer, 2012; Zamburlin et al., 2013; Henle et al., 2011; Tian et al., 2010; Wei et al., 2009; Evans and Falke, 2007; Heckman and Plummer, 2013; Schafer et al., 2012). Plasma membrane Ca^{2+} channels including transient receptor potential (TRP) and Orai1 channels, and endoplasmic reticulum Ca^{2+} channels including ryanodine and IP_3 receptors, are proposed to give rise to the leading edge Ca^{2+} influxes and effluxes, respectively (Wei et al., 2012; Tsai and Meyer, 2012; Zamburlin et al., 2013; Henle et al., 2011; Tian et al., 2010; Wei et al., 2009; Heckman and Plummer, 2013; Schafer et al., 2012). In neurite growth, plasma membrane L- and N-type voltage dependent Ca^{2+} channels may also play a role (Zamburlin et al., 2013). In general, the signaling events that trigger the local, leading edge Ca^{2+} signals in chemotaxing cells are not yet well understood, but at least in certain cell types $PI(3,4,5)P_3$ may trigger Ca^{2+} influx by directly or indirectly opening leading edge TRP channels (Henle et al., 2011). Notably, the leading edge Ca^{2+} signal is quite low amplitude (Wei et al., 2012; Tsai and Meyer, 2012; Wei et al., 2009; Evans and Falke, 2007) and easily collapsed by presence of high affinity Ca^{2+} chelators (Evans and Falke, 2007). Besides this modest leading edge Ca^{2+} signal, migrating cells typically possess a larger Ca^{2+} signal at the trailing edge of the cell where it is required for retraction from the substrate (Wei et al., 2012, 2009; Yang and Huang, 2005). Thus, although both ends of the cell possess Ca^{2+} signals, the characteristics and functions of the two polar signals are different.

Further studies are needed to define the molecular steps that couple receptor activation to PI3K activation at the leading edge membrane, and the pathway mechanisms that control the steady state levels of the substrate $PI(4,5)P_2$ and product $PI(3,4,5)P_3$ lipids during a signal. Additional key questions include the relative importance of the different Ca^{2+} channels and Ca^{2+} pools implicated in chemotaxis, how the key Ca^{2+} channels are regulated by the chemotaxis pathway, and whether multiple types of Ca^{2+} signals exist and play different roles at the leading edge – for example, influx vs efflux, or lamellapodia vs filapodia.

3. Functions of the leading edge lipid and Ca^{2+} signals

3.1. Roles of $PI(3,4,5)P_3$ and Ca^{2+}

The primary shared function of the leading edge $PI(3,4,5)P_3$ and Ca^{2+} signals is to drive the targeting of dozens of signaling proteins from cytoplasm to the leading edge membrane, yielding one of the most dramatic protein recruitments in cell biology (Fig. 1) (Jin, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011; Kolsch et al., 2008; von Philipsborn and Bastmeyer, 2007; Evans and Falke, 2007). The recruited signaling proteins each recognize and bind specific lipids on the cytoplasmic leaflet of the leading edge plasma membrane.

The diverse signaling proteins recruited from the cytoplasm to the leading edge typically possess a regulated membrane targeting domain. In most cases this targeting domain is either a pleckstrin homology (PH) domain that recognizes and binds the $PI(3,4,5)P_3$ signaling lipid, or a C2 domain that is activated by Ca^{2+} binding and then docks to lipids on the target membrane surface (Corbalan-Garcia et al., 2007; Leonard and Hurley, 2011; Newton, 2009; Lemmon, 2008; Cho, 2001; Nalefski and Falke, 1996). C2- and PH-driven targeting is a common feature of signaling pathways, and these two ubiquitous targeting motifs are found in over 620 and 330 human proteins, respectively, (Punta et al., 2012).

Typically, the C2 or PH domain-directed membrane targeting reaction serves to activate the signaling protein via enhanced proximity to key reactants in its signaling reaction (Fig. 2) (Corbalan-Garcia et al., 2007; Leonard and Hurley, 2011; Newton, 2009; Lemmon, 2008; Cho, 2001; Nalefski and Falke, 1996). Specifically, the reaction brings the signaling protein to the membrane surface where its effector and substrate molecules are located, yielding faster on-rates for the binding events essential for signaling domain activity. In some cases, direct interactions of the signaling domain with the bilayer may alter the conformation of the signaling protein or the interactions between its domains, providing additional activation.

Further studies are needed to investigate isolated reports that certain cell types migrating in specific conditions can utilize an alternative pathway, not involving $PI(3,4,5)P_3$ and/or Ca^{2+}, to control chemotaxis, although the normal pathway involving $PI(3,4,5)P_3$ and Ca^{2+} appears to be dominant in most cell types and settings (Jin, 2013; Weiger and Parent, 2012). Additional work is also needed to ascertain whether $PI(3,4,5)P_3$ and Ca^{2+} have roles beyond membrane recruitment at the leading edge, as exemplified by the plausible regulation of Ca^{2+} influx by $PI(3,4,5)P_3$-gated TRP channels (Henle et al., 2011), and the likely role of Ca^{2+} in regulating leading edge myosin activity (Tsai and Meyer, 2012).

3.2. Roles of $PI(4,5)P_2$ and DAG

While not a signaling molecule itself, the constitutive plasma membrane lipid $PI(4,5)P_2$ is essential as the substrate for production of $PI(3,4,5)P_3$ by PI3K (Jin, 2013; Vadas et al., 2011). $PI(4,5)P_2$ also plays a major role as a target lipid for the plasma membrane recruitment of various signaling proteins including the classical isoforms of protein kinase C (cPKCs) (Evans and Falke, 2007; Thapa and Anderson, 2012; Corbalan-Garcia et al., 2007; Corbin et al., 2007; Evans et al., 2006), and serves as an attachment point for cytoskeletal proteins involved in leading edge dynamics (Thapa and Anderson, 2012; Golebiewska et al., 2008). For example, the cPKC isoform $PKC\alpha$ is known to be recruited by the local Ca^{2+}

signal to the leading edge, where its C2 domain must bind both PS and PI(4,5)P$_2$ to ensure the high affinity, specific plasma membrane specificity while awaiting the appearance of a DAG signal (Evans and Falke, 2007; Corbalan-Garcia et al., 2007; Corbin et al., 2007; Evans et al., 2006).

In certain cases, mutations that enhance PI(4,5)P$_2$ targeting can cause human disease. In targeting reactions controlled by PI(3,4,5)P$_3$-specific PH domains, the PH domain must ignore the constitutive PI(4,5)P$_2$ lipid while recognizing and binding to the rare PI(3,4,5)P$_3$ signaling lipid. Certain PH domain mutations are known to convert the highly specific PI(3,4,5)P$_3$ binding site into a dual specificity PI(4,5)P$_2$/PI(3,4,5)P$_3$ binding site, causing constant PH domain targeting to plasma membrane PI(4,5)P$_2$ (Pilling et al., 2011; Landgraf et al., 2008; Carpten et al., 2007). The resulting constitutive membrane targeting yields superactivation of the signaling protein and often triggers disease. For example, the E17K mutation at the PI(3,4,5)P$_3$ binding site of PKB/AKT PH domain generates dual PI(4,5)P$_2$/PI(3,4,5)P$_3$ specificity, yielding constitutive plasma membrane targeting and superactivation of the PKB/AKT kinase domain, thereby greatly increasing the incidence of multiple human cancers and Proteus syndrome (Landgraf et al., 2008; Carpten et al., 2007; Lindhurst et al., 2011).

If phospholipase C (PLC) activity is present at the leading edge, the resulting PI(4,5)P$_2$ hydrolysis would produce both the soluble second messenger Ins(1,4,5)P$_3$ and the signaling lipid DAG (Tsai and Meyer, 2012; Abramovici et al., 2009; Thapa and Anderson, 2012). In turn, Ins(1,4,5)P$_3$ could open the IP$_3$-sensitive Ca^{2+} channels of internal stores, contributing to the local Ca^{2+} signal.

Furthermore, the DAG resulting from PLC hydrolysis of PI(4,5)P$_2$ at the leading edge would likely activate cPKCs. At least one isoform, PKCα, is known to be recruited to the macrophage leading edge by its C2 domain that associates with Ca^{2+}, PS and PI(4,5)P$_2$ (Evans and Falke, 2007; Corbin et al., 2007; Evans et al., 2006). The binding of DAG to the C1 domain is essential for full activation of the cPKC kinase domain (Newton, 2009), and for its proposed role in generating free PI(4,5)P$_2$ by phosphorylating MARCKS (see 4.4 below). In short, while direct detection of Ins(1,4,5)P$_3$ and DAG signals has not yet been achieved at the leading edge, such signals are likely to be present and essential.

Further studies are needed to test the prediction that PLC activity and localized accumulations of Ins(1,4,5)P$_3$ and DAG are essential players in leading edge signaling, and to determine which PLC isoforms are involved in leading edge PI(4,5)P$_2$ hydrolysis and their mechanisms of regulation.

4. The assembled leading edge signaling circuit

4.1. Role of surface binding and 2-D diffusion

Together the dozens of signaling proteins recruited to the leading edge by PI(3,4,5)P$_3$ and Ca^{2+} signals form a complex, dynamic, signaling network on the membrane surface. The lipid bilayer plays a central role in this network by serving as a shared platform for two-dimensional diffusion, thereby enabling the membrane-bound signaling proteins to

encounter and bind their membrane-associated, lipid and/or protein effectors and substrates (Ziemba et al., 2013; Ziemba and Falke, 2013; Ziemba et al., 2012; Knight et al., 2010; Knight and Falke, 2009). The resulting, membrane-localized signaling circuit controls actin polymerization, membrane remodeling, and other dynamic processes essential for growth of the leading edge up attractant gradients. The many important components of this network are too numerous to consider herein, but the reader is referred to extensive reviews (Jin, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011; Kolsch et al., 2008; von Philipsborn and Bastmeyer, 2007; Evans and Falke, 2007).

4.2. An essential leading edge positive feedback loop

A key feature of the leading edge signaling circuit is a positive feedback loop formed by a subset of the signaling proteins recruited to the leading edge (Jin, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011; Hawkins et al., 2010; Swaney et al., 2010; Kolsch et al., 2008; Mortimer et al., 2008; von Philipsborn and Bastmeyer, 2007; Bourne and Weiner, 2002; Evans and Falke, 2007; Charest and Firtel, 2006). After this positive feedback loop is established it amplifies both activating and inhibitory signals, such that the activation of an individual loop component yields upregulation of all other loop components. At the other extreme, inhibition of any loop component yields downregulation of all other loop components. The positive feedback loop has been shown to be essential for maintenance of a stable, ruffling leading edge possessing a very high density of signaling proteins, actin mesh and other cytoskeletal elements. The resulting active leading edge region often becomes a semi-permanent structural and functional region of the cell, approaching an organelle in its complexity and stability, but of course lacking a complete membrane boundary.

It is hypothesized that the positive feedback loop plays a central role in the compass that determines the direction of leading edge movement up the attractant gradient (Cai and Devreotes, 2011; Evans and Falke, 2007; Charest and Firtel, 2006). In this model, when a filopodium grows up the attractant gradient the positive feedback loop amplifies the growing attractant signal and speeds filopodial growth in this favorable direction. By contrast, when a filopodium grows perpendicular to the gradient or down the gradient, the positive feedback loop detects either no attractant change or an attractant decrease, and responds by ignoring or perhaps even slowing filopodial growth in this counterproductive direction.

4.3. Elements of the leading edge positive feedback loop

It has become apparent that the positive feedback loop has many essential components. Strong evidence exists that PI3K, PI(3,4,5)P$_3$, filamentous actin, Ca^{2+}, PKCα, Ras GTPase and Rho GTPase are central components (Jin, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011; Hawkins et al., 2010; Collins and Meyer, 2009; Wei et al., 2012; Tsai and Meyer, 2012; Zamburlin et al., 2013; Henle et al., 2011; Swaney et al., 2010; Kolsch et al., 2008; Mortimer et al., 2008; von Philipsborn and Bastmeyer, 2007; Tian et al., 2010; Wei et al., 2009; Evans and Falke, 2007). The Ca^{2+} signal, missing in earlier models, is now a central feature. Fig. 3 presents both confirmed components and unconfirmed, but plausible, elements in a preliminary working model for the leading edge pathway and its positive feedback circuit.

Further studies are needed to test and fill in the gaps of this working circuit diagram (Fig. 3), which remains speculative and is undoubtedly missing important components and regulatory interactions. For example, much has been learned about the proteins that regulate actin remodeling (Heckman and Plummer, 2013; Insall and Machesky, 2009), but less is known about the machinery that controls membrane remodeling. The connections of the feedback loop to these remodeling systems must be defined and incorporated into the model.

4.4. Working model for the regulation of PKCα, PI3K and PDK1 in the leading edge positive feedback loop

Beyond the circuit diagram of the positive feedback loop, much remains to be learned about the structural and mechanistic underpinnings of information flow and regulation within the loop. Fig. 4 illustrates a working model proposing plausible, but largely untested, regulatory interactions between the three master kinases PKCα, PI3K and PDK1. These interactions are hypothesized to provide both positive feedback and an internal brake that prevents runaway loop activation.

This small section of the feedback loop is proposed to begin with Ca^{2+}- and DAG-activated, membrane-bound PKCα docked to target lipids PS, $PI(4,5)P_2$ and DAG (Evans and Falke, 2007; Corbalan-Garcia et al., 2007; Corbin et al., 2007; Evans et al., 2006; Newton, 2009). PKCα is known to phosphorylate the membrane binding peptide of the MARCKS protein (McLaughlin et al., 2002). Unphosphorylated MARCKS tightly binds multiple (~3) $PI(4,5)P_2$ molecules and effectively sequesters them. The model supposes that unmodified MARCKS maintains the density of free, leading edge $PI(4,5)P_2$ at a level sufficient to recruit PKCα via its C2 domain, but low enough to limit the activity of the lipid kinase PI3K and the lipid phosphatase PTEN which require $PI(4,5)P_2$ as a substrate and a target lipid, respectively. When PKCα modifies MARCKS, each phosphorylation event is believed to release a free $PI(4,5)P_2$ molecule in a stepwise fashion (Golebiewska et al., 2008; McLaughlin et al., 2002; Wang et al., 2004; Gambhir et al., 2004). The resulting rise in free $PI(4,5)P_2$ levels increases the substrate availability and lipid kinase activity of PI3K, which phosphorylates $PI(4,5)P_2$ yielding $PI(3,4,5)P_3$. This PI3K activity decreases the leading edge $PI(4,5)P_2$, which can also be degraded by phospholipase C activity to generate leading edge DAG and $Ins(1,4,5)P_3$ (Jin, 2013; Wei et al., 2012; Tsai and Meyer, 2012; Vadas et al., 2011; Wei et al., 2009; Evans and Falke, 2007). MARCKS is proposed to buffer the $PI(4,5)P_2$, thereby inhibiting these degradation reactions while being fully reversible by PKCα activity.

Following activation of the PI3K, the product $PI(3,4,5)P_3$ diffuses away from the PI3K and recruits PH domains to the plasma membrane. One such PH domain is that of PDK1, which is recruited by the $PI(3,4,5)P_3$ signal to the leading edge membrane. Subsequently, the $PI(3,4,5)P_3$-occupied, membrane-bound PH domain plays a central role in the activation of the PDK1 catalytic domain and the stimulation of its protein kinase activity (Ziemba et al., 2013; Masters et al., 2010).

The indicated flow of information can contribute to positive feedback since the first and last steps are cascade-type activation events: (i) a single activated PKCα protein kinase molecule generates many free $PI(4,5)P_2$ molecules (amplifying cascade), then (ii) each $PI(4,5)P_2$

molecule can be converted by PI3K into a $PI(3,4,5)P_3$ molecule (no amplification), then (iii) each $PI(3,4,5)P_3$ molecule can recruit a PDK1 molecule to the membrane (no amplification), and finally (iv) each membrane-activated PDK1 kinase molecule phosphorylates many substrate proteins (amplifying cascade).

It is hypothesized that membrane-bound, active $PKC\alpha$ and PDK1 form a stable, membrane-associated complex that may inhibit both enzymes, thereby yielding an activity brake ensuring that the positive feedback signal does not grow in an uncontrolled way, ultimately yielding a runaway pathway. The activation loops of cPKCs are phosphorylated by PDK1 early in their cellular processing, thus $PKC\alpha$ bound to leading edge is already phosphorylated by PDK1 (Newton, 2010). In the cytoplasm, both unphosphorylated and phosphorylated $PKC\alpha$ form inactive complexes with PDK1 that are sufficiently stable to be isolated by immunoprecipitation (Le Good et al., 1998). The hypothesized membrane-bound, $PKC\alpha$-PDK1 complex has not yet been detected but is plausible given the stability of the soluble complex.

Further studies are needed to test and further refine the proposed protein regulatory interactions in the $PKC\alpha$ – PI3K – PDK1 branch of the leading edge positive feedback loop. The current working model (Fig. 4) is presented as a starting point to facilitate such testing.

5. Direct PIP lipid-Ca^{2+} interactions and PIP lipid clustering

Multiple studies, reviewed in this issue by Janmey et al. (Wang et al., 2013), have suggested that sufficient mole densities of $PI(4,5)P_2$ or $PI(3,4,5)P_3$ lipid in a bilayer, together with sufficient concentrations of Ca^{2+}, can lead to a direct interaction between multiple PIP lipids and Ca^{2+}. Under certain conditions, this direct interaction can lead to Ca^{2+}-induced clustering of PIP lipids in bilayers. To facilitate detection of clusters *in vitro,* the PIP lipid mole densities and free Ca^{2+} concentrations typically employed in such studies are significantly higher than physiological levels. Much remains to be learned about the prevalence and significance of Ca^{2+}-induced PIP clusters in cells.

In principle, specific protein–lipid interactions could stabilize Ca^{2+}-induced, PIP lipid clusters, enabling their formation at more physiological lipid and Ca^{2+} levels. For example, the $PI(4,5)P_2$ binding site of $PKC\alpha$ can bind a single $PI(4,5)P_2$ molecule, but when saturated is occupied by two $PI(4,5)P_2$ molecules (Lai et al., 2010; Corbalan-Garcia et al., 2003). In the latter case, MD simulations indicate the two highly anionic $PI(4,5)P_2$ molecules are bound side-by-side in a pairwise cluster stabilized by Ca^{2+} and multiple positive charges provided by the protein (Lai et al., 2010). Thus, during a cellular Ca^{2+} signal a portion of the membrane-bound $PKC\alpha$ population will be associated with Ca^{2+}-stabilized $PI(4,5)P_2$ pairs. If physiological conditions produce membrane regions containing both $PI(4,5)P_2$ clusters and PS, such regions could exhibit increased affinity for cPKC C2 domains that require both $PI(4,5)P_2$ and PS for optimal target membrane affinity and specificity (Evans and Falke, 2007; Corbalan-Garcia et al., 2007; Corbin et al., 2007; Evans et al., 2006). Similarly, PH domains require both $PI(3,4,5)P_3$ and PS for high affinity, rapid target membrane acquisition (Corbin et al., 2004; Knight and Falke, 2009; Lai et al., 2013), thus their targeting reactions could be facilitated by regions containing both $PI(3,4,5)P_3$ clusters and PS.

Further studies are needed to ascertain whether Ca^{2+}-induced, PIP lipid clusters occur in cells, to determine their sizes and lipid compositions, and to elucidate their effects on the crucial C2- and PH-domain driven membrane targeting reactions that control cell migration and many other cellular processes.

Acknowledgments

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Abbreviations

PH domain	pleckstrin homology domain
C1A and C1B	first conserved domains of classical protein kinase C
C2	second conserved domain of classical protein kinase C
PKC	protein kinase C
cPKC	one of the classical isoforms of protein kinase C
PI3K	phosphoinositide 3-kinase
f-Actin	filamentous actin
PDK1	phosphoinositide-dependent kinase isoform 1
PLC	phospholipase C
Ras	a small GTPase or G-protein of the Ras family
Rho	a small GTPase or G-protein of the Rho family
GRP1	general receptor for phosphoinositides isoform 1
PKB/AKT	protein kinase B, also known as AKT
ARF6	ADP ribosylation factor 6, a small G-protein
MARCKS	myristoylated alanine-rich C-kinase substrate
PTEN	phosphatase and tensin homologue
GPCR	G-protein coupled receptor
RTK	receptor tyrosine kinase

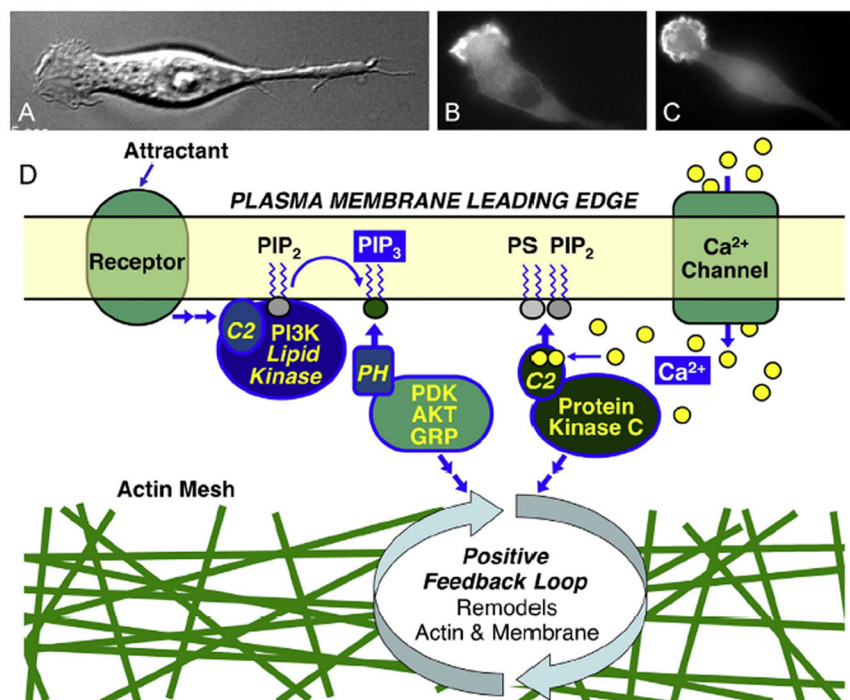|	
PS	phosphatidylserine
PI(4,5)P$_2$ or PIP$_2$	phosphatidylinositol 4,5-bisphosphate
PI(3,4,5)P$_3$ or PIP$_3$	phosphatidylinositol 3,4,5-trisphosphate
Ins(1,4,5)P$_3$ or IP$_3$	inositol 1,4,5-trisphosphate
DAG	diacylglycerol

Fig. 1.

Second messenger signals at the leading edge of polarized ameboid cells (Evans and Falke, 2007). Shown are polarized RAW macrophages illustrating (A) the ruffling leading edge in a phase contrast image, (B) the local Ca^{2+} signal revealed by YFP-PKCα recruited to the leading edge membrane in a wide field fluorescence image, and (C) the local PI(3,4,5)P$_3$ signal revealed by GFP-PKB/AKT-PH domain recruited to the leading edge membrane in a wide field fluorescence image. (D) Schematic overview of parallel Ca^{2+} and PI(3,4,5)P$_3$ (PIP$_3$) signals that activate PH and C2 membrane targeting domains, triggering recruitment of signaling proteins to the leading edge membrane (Jin, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011; Kolsch et al., 2008; von Philipsborn and Bastmeyer, 2007; Evans and Falke, 2007; Corbalan-Garcia et al., 2007; Leonard and Hurley, 2011; Newton, 2009; Lemmon, 2008; Cho, 2001; Nalefski and Falke, 1996). The resulting bilayer-associated signaling pathway includes a positive feedback loop that controls actin and membrane remodeling essential for leading edge structure, stability, dynamics, and directed cell migration (Jin, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011; Hawkins et al., 2010; Swaney et al., 2010; Kolsch et al., 2008; Mortimer et al., 2008; von Philipsborn and Bastmeyer, 2007; Bourne and Weiner, 2002; Evans and Falke, 2007; Charest and Firtel, 2006).
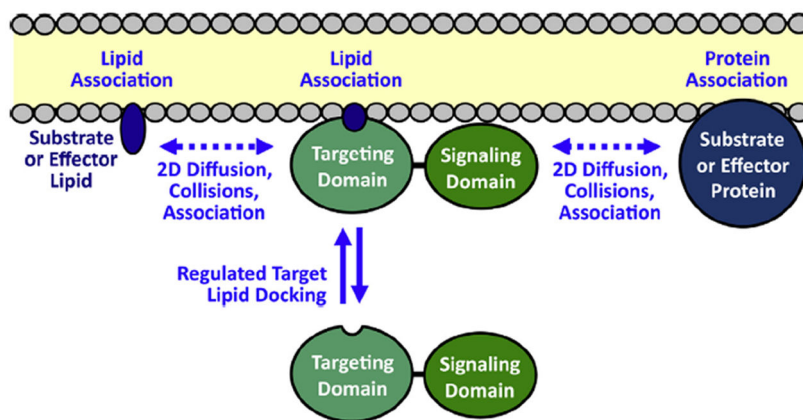
Fig. 2.

Function of PH and C2 membrane targeting domains in the recruitment and activation of signaling proteins on target membrane surfaces (Corbalan-Garcia et al., 2007; Leonard and Hurley, 2011; Newton, 2009; Lemmon, 2008; Cho, 2001; Nalefski and Falke, 1996). First, a 2° messenger signal triggers the recruitment of the signaling protein targeting domain from the cytoplasm to the bilayer surface: many PH domains are recruited to the plasma membrane by a PI(3,4,5)P$_3$ (PIP$_3$) lipid signal, while the C2 domains of classical protein kinase C isoforms (cPKCs) are activated by Ca^{2+} binding and then dock to PS and PI(4,5)P$_2$ (PIP$_2$) on the plasma membrane. Second, the signaling domain of each recruited protein is activated by its proximity on the membrane surface to membrane-associated protein and/or lipid molecules that serve as effectors or substrates. At the leading edge membrane, PIP$_3$ and Ca^{2+} both recruit signaling proteins and establish a signaling network on the bilayer surface. Lateral, 2-dimensional diffusion on the bilayer is required for the signaling proteins to encounter and bind their signaling partners, and for the resulting membrane-bound products to diffuse away. Thus, the lipid bilayer serves as a 2-dimensional platform for the assembly of the dynamic signaling circuit, and for the diffusion of its many components during signaling reactions.
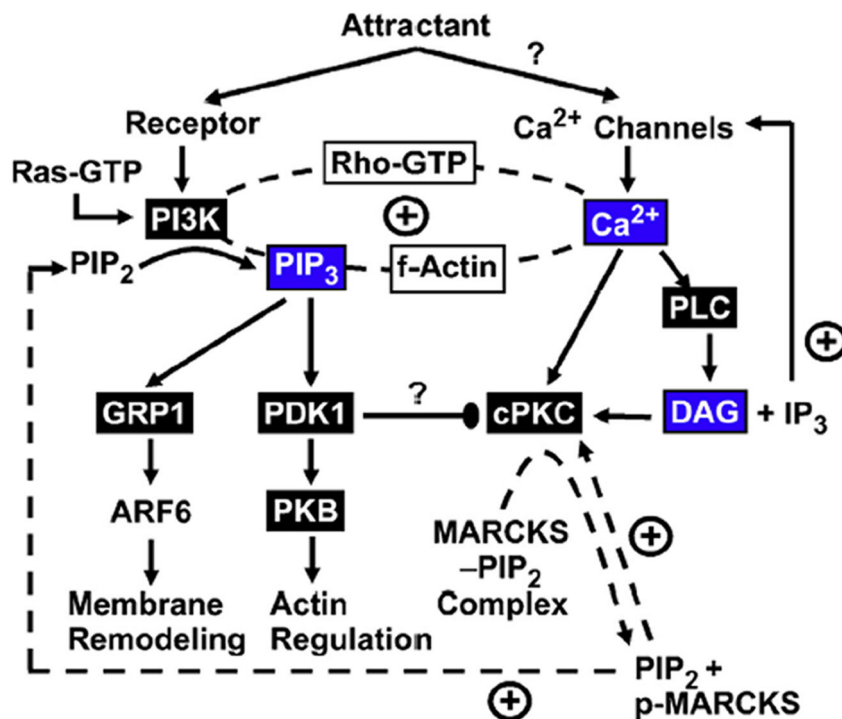
Fig. 3.
Schematic, partial model of the intricate leading edge signaling circuit, illustrating known and hypothesized components and their interactions (Jin, 2013; Weiger and Parent, 2012; Cai and Devreotes, 2011; Hawkins et al., 2010; Collins and Meyer, 2009; Wei et al., 2012; Tsai and Meyer, 2012; Zamburlin et al., 2013; Henle et al., 2011; Swaney et al., 2010; Kolsch et al., 2008; Mortimer et al., 2008; von Philipsborn and Bastmeyer, 2007; Tian et al., 2010; Wei et al., 2009; Evans and Falke, 2007). Attractant signals switch on receptors and the Ras protein, which recruit and activate the lipid kinase activity of PI3K that phosphorylates the constitutive lipid $PI(4,5)P_2$ (PIP$_2$) to generate the signaling lipid $PI(3,4,5)P_3$ (PIP$_3$). Attractant signals may also activate plasma membrane Ca^{2+} channels that generate an essential Ca^{2+} influx at the leading edge. The $PI(3,4,5)P_3$ and Ca^{2+} second messengers are essential players in the leading edge positive feedback loop (dashed line) along with one or more small GTPases of the Rho family, filamentous actin and PKCα. The $PI(3,4,5)P_3$ signal recruits PDK1 to the leading edge where it is activated and stimulates branches leading to membrane and actin remodeling needed for leading edge expansion up the attractant gradient. The Ca^{2+} signal activates and recruits PKCα to the leading edge membrane where it is proposed to phosphorylate the $PI(4,5)P_2$-sequestration region of MARCKS protein, which in turn is predicted to increase the free $PI(4,5)P_2$ density and thereby activate PI3K and self-activate PKCα. Ca^{2+} may also activate PLC to generate a putative DAG lipid signal. Finally, PDK1 phosphorylation of PKCα in solution is essential for maturation and activation of the latter. On the membrane surface PDK1 and PKCα are hypothesized to combine and form a stable, inactive complex that could act as a brake to prevent runaway positive feedback. Proteins in boxes contain a PH domain (black box) or a C2 domain (white box). Second messengers are also highlighted (blue box).
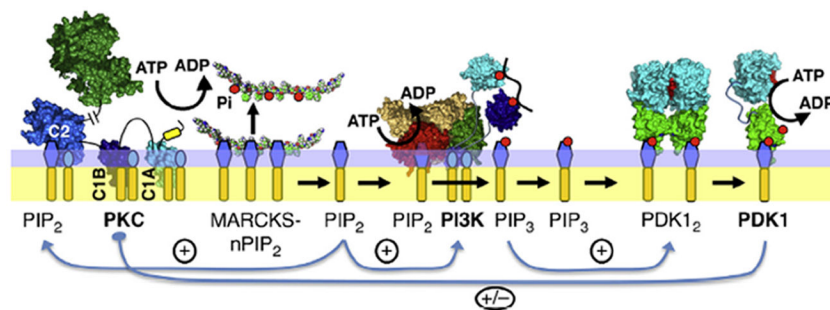
Fig. 4.

Working model for the molecular interactions between the activities of PKCα, PI3K and PDK1 in the leading edge positive feedback loop. Shown are the three master kinases and their effector lipids on the cytoplasmic leaflet of the leading edge membrane. Kinase-active PKCα is bound via its Ca^{2+}-occupied C2 domain to PS and PI(4,5)P_2 (PIP$_2$), and via its C1A and C1B domains to PS and DAG (Evans and Falke, 2007; Corbalan-Garcia et al., 2007; Corbin et al., 2007; Evans et al., 2006; Newton, 2009). This active PKCα is proposed to phosphorylate the PI(4,5)P_2-sequestration region of MARCKS protein, thereby releasing PI(4,5)P_2 from MARCKS and increasing the local free PI(4,5)P_2 density at the leading edge (Golebiewska et al., 2008; McLaughlin et al., 2002; Wang et al., 2004; Gambhir et al., 2004). The newly released PIP$_2$ helps counteract the loss of PI(4,5)P_2 due to the enzymatic activities of PI3K and PLC (Jin, 2013; Wei et al., 2012; Tsai and Meyer, 2012; Vadas et al., 2011; Wei et al., 2009; Evans and Falke, 2007), and this PI(4,5)P_2 release activates both PI3K that requires PI(4,5)P_2 as a substrate, and PKCα that requires PI(4,5)P_2 for stable plasma membrane association. PI3K converts PI(4,5)P_2 to the signaling lipid PI(3,4,5)P_3 (PIP$_3$), which recruits many signaling proteins including PDK1 to the leading edge membrane. PDK1 is an inactive dimer that is converted on the membrane surface to an active monomer (Ziemba et al., 2013; Masters et al., 2010). The resulting PDK1 monomer may form an inhibitory complex with PKCα that has been isolated from cytoplasm but not yet investigated on a membrane surface (Le Good et al., 1998). Further studies are needed to test the proposed interactions between the three master kinases, and the roles of these interactions in leading edge positive feedback and signal amplification.