Minireview

The role of phosphatidic acid in the regulation of the Ras/MEK/Erk signaling cascade

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Abstract Phosphatidic acid (PA) is an important second messenger produced by the activation of numerous cell surface receptors. Recent data have suggested that PA regulates multiple cellular processes. This review addresses primarily the role of PA in the regulation of the Erk1/2 cascade pathway. A model for the regulation of Erk1/2 phosphorylation by cell surface receptors is presented. According to this model, agonists stimulate the binding of GTP to Ras and the activation of phospholipase D to generate phosphatidic acid. PA promotes the binding of cRaf-1 kinase to the membrane, where it interacts with Ras.GTP and other regulatory components of the pathway. Ras–Raf complexes remain bound to the surface of endosomes, where scaffolding complexes involving Ras, cRaf-1, MEK and Erk are formed. Complete activation and coupling of the cascade requires endocytosis, a process that is also modulated by PA.

Key words: Phosphatidic acid; Phospholipase D; Signaling cascade; Lipid second messenger

1. Phosphatidic acid as a second messenger

Phosphatidic acid (PA) is an important metabolite involved in phospholipid biosynthesis and membrane remodeling. However, recent data have suggested that PA may play a crucial role in the regulation of several important biological events. For instance, PA has been implicated in the regulation of protein phosphorylation [1–8], in the activation of oxidative processes [9–11], and in the modulation of membrane traffic [12–14]. Many of these processes are mediated by a previously unsuspected role of PA: the binding of PA in a highly selective and specific manner. Thus, PA appears to function in a manner similar to many other lipid second messengers: by promoting the binding of selected targets to specific regions of the cell membrane.

The direct interaction of PA with cell proteins has been demonstrated in a small group of proteins. Thus, the interactions of PA with the protein kinase cRaf-1 have been mapped to a 35 amino acid stretch in the CR3 domain of the protein [2,5], and the binding of mTOR seems to involve R2109 near the rapamycin-binding domain of the protein [1]. Likewise, the binding of PA to the cAMP-specific phosphodiesterase PDE4D3 seems to be mediated by amino acids 31–59, located within the regulatory domain of the protein [15], and the PA binds to residues contained within the 41 C-terminal amino acids of the tyrosine protein phosphatase SHP-1 [16]. Fig. 1 shows the sequences of the putative binding sites of Raf, KSR, mTOR, PDE4D3 and SHP-1.

Examination of these putative PA-binding domains does not reveal great similarity among these sequences, except for the fact that they all contain at least one polybasic motif. In the case of cRaf-1 and mTOR, this polybasic motif is flanked by one or two hydrophobic stretches. The PA-binding region of SHP-1 is located in the C-terminal end of the protein, in a long hydrophilic stretch that contains 15 basic and six acidic residues. Finally, the putative PA-binding region of PDE4D3 is characterized by two histidine-rich stretches. The identification of consensus PA-interacting motifs does not appear feasible at this time given the small number of putative target sequences. More recent work has demonstrated the existence of two PA-binding sites within the PX domain of p47phox (L. McPhail, personal communication). Although no information on the actual PA-binding sites is available at this time, the PX domain of p47phox contains one RR pair at the end of a hydrophobic stretch that bears similarity to the PA-binding regions of Raf-1 and mTOR, suggesting this as a common motif for at least a sub-class of PA-binding proteins.

However, there is little doubt that PA plays a significant role in the regulation of the function of these proteins. The

Raf-1	390	FRNEVAVLRKTRHVNILLFMGYMTKDNLAIVTQWCEG	426
A-raf	349	FKNEMQVLRKTRHVNILLFMGFMTRPGFAIITQWCEG	385
B-raf	497	FKNEVGVLRKTRHVNILLFMGYSTKPQLAIVTQWCEG	533
KSR	604	FKKEVMNYRQTRHENVVLFMGACMNPPHLAIITSFCKG	641
mTOR 2	101	WDLYYHVFRRISKQLPQLTSLELQYVSPKLLMCRDLE	2137
SHP-1	558	SSKHKEDVYENLHTKNKREEKVKKQRSADKEKSKGSLKRK	597
PDE4D3	31	HLWRHEQHHQYPLRQPQFRL	50

Fig. 1. The putative PA-binding domains of Raf-1, A-Raf, B-Raf, KSR, mTOR, SHP-1 and PDE4D3. Raf-1, A-Raf and B-Raf are protein kinases of the Raf family. KSR is the kinase suppressor of Ras, a putative scaffolding protein. mTOR (target of rapamycin) is a protein kinase related to the PI3 kinase superfamily. SHP-1 (or PTP1C) is a protein tyrosine phosphatase. PDE4D3 is one of the isoforms of cAMP-specific phosphodiesterase.

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binding of PA to SHP-1, for instance, activates the phosphatase in vitro [16], and the data clearly indicate that the interactions with PA are essential for the recruitment of cRaf-1 to membranes [5].

2. Receptor-mediated regulation of PA production

PA can be generated by several cellular processes: (a) the hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD); (b) the phosphorylation of diacylglycerol (DAG) by DAG kinase; and (c) the acylation of lyso-PA by lyso-PA acyl transferases. All of these processes are regulated at some level by extracellular signals. The regulation of PA levels by modulation of the activity of PLD and by the regulation of DAG kinase has been reviewed extensively [17–19].

PLD activity is stimulated by many cell surface receptors, including receptor tyrosine kinases, G protein-coupled receptors and cytokine receptors (see the review by Exton [19]). Mammalian cells express two isoforms of PLD, PLD1 and PLD2. Overexpression of either isoform increases agonist-dependent PLD responses in model systems. However, only the overexpression of a catalytically inactive mutant of PLD2 (K758R-PLD2) appears to inhibit PLD.5 activation by insulin or PDGF in HIRcB fibroblasts [20,21], or by angiotensin II and PDGF in vascular smooth muscle cells [22,23]. In these systems, the overexpression of the catalytically inactive K898R-PLD1 was without effect.

These data suggest that PLD2 is the main target for receptor-dependent PLD activation. The dominant negative effects of K758R-PLD2 have provided one of the few tools available to study the role of PA and PLD activation in cellular processes. In fact, the only pharmacological inhibitors of PLD available to date are short-chain primary alcohols, which actually function as alternative substrates to water in the transphosphatidylation reaction catalyzed by PLD. Therefore, since the inhibition of PA formation by primary alcohols is accompanied by the formation of phosphatidylalcohols, the data obtained using these inhibitors are always difficult to interpret.

3. The regulation of protein phosphorylation by PA

Several studies have suggested that PA may regulate specific kinase cascades. For instance, recent work on the mTOR signaling cascade has shown that 1-butanol, but not 2-butanol, inhibits serum-stimulated activation of S6 kinase, one of the main targets of mTOR [1]. Likewise, treatment with ethanol inhibits phorbol ester-induced translocation of cRaf-1 to membranes [2]. In vitro studies have demonstrated that there are at least two-protein kinase activities regulated by PA in neutrophils. The protein tyrosine kinase Fgr [7] and a novel protein kinase have been implicated in these activities [4]. Likewise, the PKC isoform PKC-ζ appear to be activated by direct interactions with PA [3]. However, the best characterized functional role of PA in the regulation of protein phosphorylation cascades remains the regulation of the Ras/Raf/MEK/Erk cascade.

The protein kinase cRaf-1 is one of the main targets of the small GTPase Ras. Ras is a membrane-bound protein; therefore, it has long been argued that cRaf-1 activation requires its translocation to the membrane. According to the current models of cRaf-1 activation, Ras–Raf interactions play a central role in the translocation of cRaf-1 to the membrane. However, the binding of activated Ras.GTP to cRaf-1 is only part of the story. cRaf-1 is phosphorylated in serine, threonine and tyrosine residues by specific enzymes, and these phosphorylation reactions appear to be essential for the activation of the kinase (see [24] for a review). The idea that PA is a regulator of the Erk phosphorylation cascade was first proposed by Ghosh et al. in 1996 [2]. Ghosh et al. reasoned that, since cRaf-1 must interact with the membrane in order to be activated, then the interaction with specific phospholipids may contribute significantly to the stabilization of cRaf-1–membrane complexes [2]. They went on to show that cRaf-1 interacts with phosphatidylserine in a highly selective manner [2,25]. However, during these studies, a second lipid-binding site, specific for PA, was detected in the CR3 domain of cRaf-1 [2]. Mutational analysis determined that the PA-binding site was contained within a 35 amino acid fragment within the CR3 region of cRaf-1, downstream of the ATP-binding site of the kinase [2].

The relative importance of Ras and PA binding in the translocation of cRaf-1 to membranes was studied in detail more recently. Rizzo et al. demonstrated that PA binding rather than Ras–Raf interactions drives the translocation of cRaf-1 to the membrane [5]. This was done by a combination of studies in which PA production or the binding of cRaf-1 to PA were impaired by molecular techniques. In summary, these studies showed that the blockade of PA production by expression of a dominant negative PLD2 mutant inhibits insulin-dependent translocation of cRaf-1 to the membrane and Erk1/2 phosphorylation, and that addition of PA reverted the inhibitory effects of the dominant negative PLD2 [6]. PA promoted the translocation of cRaf-1 to membranes, but failed to stimulate Raf kinase activity in vitro [6]. Furthermore, the mutation of a single residue of the polybasic motif of cRaf-1 within the putative PA-binding domain (R398A) was sufficient to reduce the affinity of the protein for PA, to impair the binding of the mutant protein to membranes, and to interfere with insulin-dependent phosphorylation of Erk1/2 [5]. In fact, the effects of this mutation of the PA-binding site on the translocation of cRaf-1 to membranes were significantly more dramatic than those of a mutation of a specific residue in the Ras-binding domain of cRaf-1 that significantly reduced Ras–Raf interactions (R89L). Finally, the overexpression of a construct containing the putative PA-binding domain of cRaf-1 fused to *Aequorea victoria* green fluorescent protein blocked insulin-dependent Erk1/2 phosphorylation in HIRcB fibroblasts [5]. More recently, detailed in vitro studies have confirmed that specific lipid interactions rather than the binding of Ras drive the binding of B- and cRaf-1 to membranes [26].

These studies therefore suggest that PA production plays a central role in the activation of the Erk pathway. The model that arises from these observations states that the translocation of cRaf-1 to the membrane is primarily driven by the binding of PA. Further studies in other model systems have confirmed at least in part the validity of this model. For instance, the expression of K758R-PLD2 in vascular smooth muscle cell lines and primary cultures has been shown to inhibit the production of PA by angiotensin II and PDGF [23,22]. This inhibition was accompanied by a decrease in agonist-induced MEK and Erk phosphorylation, and the effects of K758R-PLD2 were reversed by the addition of PA.

4. PA, endocytosis and scaffolding

Some more recent work has suggested that the binding of cRaf-1 to the plasma membrane is insufficient for the activation of the Erk1/2 cascade. In fact, the data indicate that endocytosis is essential in the mechanism of regulation of Erk1/2 phosphorylation by cell surface receptors. The evidence supporting this view arises from several sources. For instance, dynamin-dominant negative mutants block endocytosis and uncouple the Erk1/2 phosphorylation pathway [27–29]. Likewise, the blockade of endocytosis with inhibitors such as cytochalasin D also results in the inhibition of receptor-dependent Erk1/2 phosphorylation. More recent work has shown that treatment with cyclodextrin, a reagent that scavenges cholesterol from the plasma membrane, blocks the traffic of Ras and cRaf-1 and the phosphorylation of MEK and Erk1/2 without affecting the translocation of cRaf-1 to the membrane and the subsequent activation of its kinase activity [30].

Recent work has established a direct link between the generation of PA and the regulation of endocytosis. PA and other acidic phospholipids facilitate the binding of dynamin to membranes [31]. However, the role of PA in vesicle traffic appears to be much more general than the modulation of endocytosis. PA has also been shown to facilitate the binding of AP-2 and clathrin coats to lysosomal membranes [32]. Endophilin A1, a protein that plays a very important role in the recycling of synaptic vesicles, has been shown to have a lyso-PA acyl transferase activity [33]. PA also seems to play a major role in Golgi traffic, whether it is produced by PLD [13,34] or by acylation of lyso-PA [35]. In general, PA appears to facilitate the fission of vesicles. This function of PA appears to be a consequence of the selective interaction of the lipid with specific target proteins, but given the peculiar structure of PA molecules (a lipid with a small head group and two bulky fatty acid chains attached to the glycerol), it has been proposed that PA may facilitate the formation of local regions of negative curvature on cell membranes [36,37].

Independently of the specific details of the phenomenon, some recent work has shown that PA is enriched in endosomes [5]. Ghosh et al. determined the stoichiometry of PA binding to cRaf-1 to be between three and six [2]. If this stoichiometry is correct, simple calculations show that a two-fold enrichment in the mol fraction of PA on endosomes

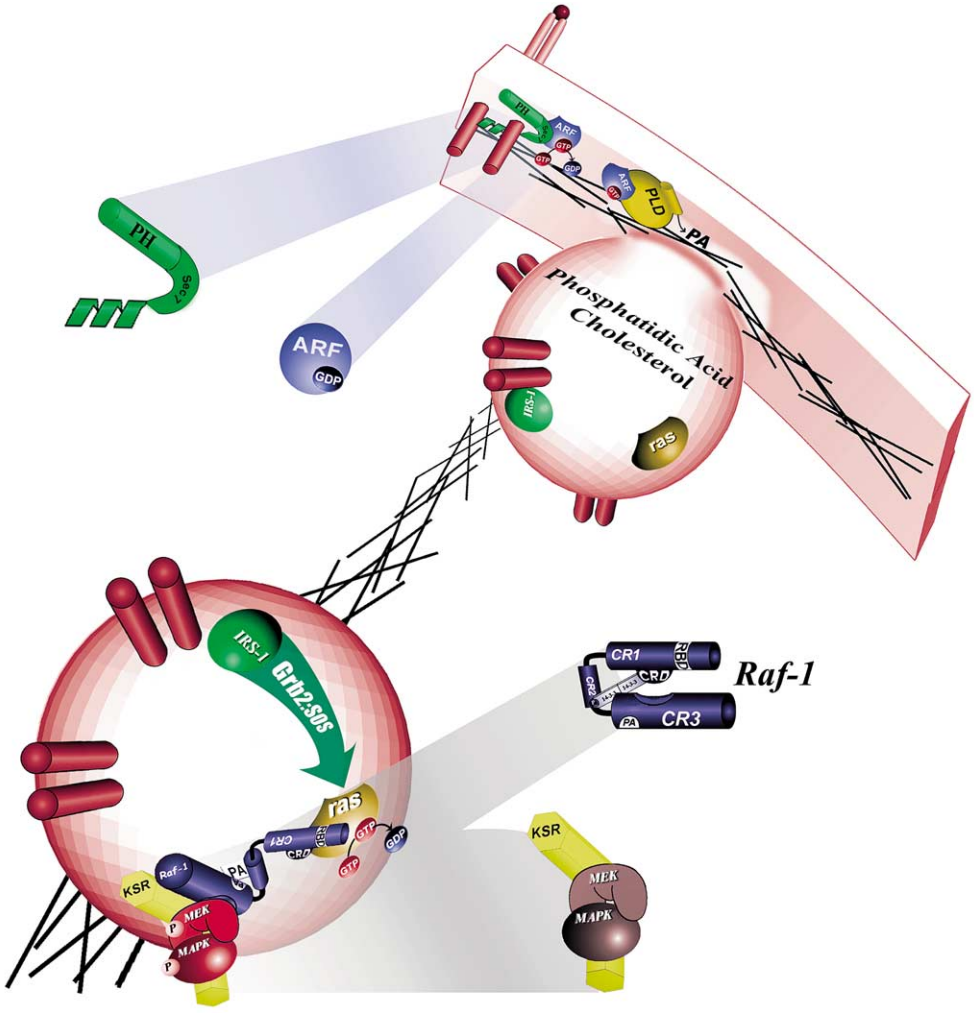

Fig. 2. A model for the role of phosphatidic acid in the regulation of the Erk1/2 cascade. Cell surface receptors activate PLD and Ras via independent, parallel mechanisms. PA promotes the binding of cRaf-1 to the plasma membrane. PA also facilitates the formation of endocytic vesicles. Ras–Raf complexes and specific scaffolding proteins that carry MEK and Erk1/2 accumulate on the surface of these endosomes. MEK and Erk1/2 phosphorylation occurs on the surface of these vesicles.

would lead to an eight- to 64-fold increase in the apparent affinity of cRaf-1 for the surface of the endosome, therefore explaining why shortly after agonist stimulation most of cRaf-1 is found bound to the surface of endosomes [5]. Interestingly, the protein KSR (Kinase Suppressor of Ras), recently shown to be a scaffolding protein that binds MEK and Erk1/2 [38,39], contains a fragment with greater than 90% homology to the PA-binding region of cRaf-1. We propose, therefore, that PA plays a central role in the accumulation of signaling and scaffolding proteins on the surface of endosomes, and that such a role is essential for the coupling of the Erk1/2 kinase cascades.

A working model based on these ideas is depicted in Fig. 2. In this model, cell surface receptors activate two signaling pathways, one leading to the activation of Ras, and the second to the activation of ARF or Rho family proteins. Activated ARF and/or Rho causes the activation of PLD2 and the local accumulation of PA. PA induces the recruitment of cRaf-1 to the plasma membrane, where it interacts with Ras and becomes activated. PA also catalyzes the formation of endocytic vesicles. Ras–Raf complexes traffic with these endosomes. Scaffolding proteins, such as KSR, are then recruited to the endosome by direct interactions with PA or other regulatory molecules. These scaffolding proteins carry with them MEK and Erk, therefore catalyzing the coupling of the phosphorylation cascade. This model is consistent with our observations and those of many other laboratories. Further testing will be required to confirm these ideas.

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