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Minireview

The regulation of phospholipase D by inositol phospholipids and small GTPases

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Abstract Phospholipase D1 and D2 (PLD1, PLD2) both have PX and PH domains in their N-terminal regions with these inositol lipid binding domains playing key roles in regulating PLD activity and localisation. The activity of PLD1 is also regulated by protein kinase C and members of the Rho and Arf families of GTPases. Each of these proteins binds to unique sites; however, there appears to be little in vitro discrimination between individual family members. In agonist-stimulated cells, however, there is specificity, with, for example in RBL-2H3 cells, antigen stimulating the activation of PLD1 by association with Arf6, Rac1 and protein kinase Cα. PLD2 appears to be less directly regulated by GTPases and rather is primarily controlled through interaction with phosphatidylinositol 4-phosphate 5-kinase that generates the activating phosphatidylinositol 4,5-bisphosphate.

Key words: Phospholipase D; Phosphatidylinositol 4,5-bisphosphate; Rac1; Arf6; Phosphatidylinositol 4-phosphate 5-kinase

1. Introduction

In most cell types a range of growth factors, hormones, cytokines and other agonists stimulate phospholipase D (PLD) catalysed phosphatidylcholine hydrolysis to generate phosphatidic acid (PtdOH) as the lipid product [1]. PtdOH apparently signals by interacting with specific binding domains in target proteins, e.g. PDE-4A1 [2], PP1 [3], thereby leading to the stimulation of physiological responses. These include the modulation of membrane trafficking, e.g. exocytosis [4] and endocytosis [5], and reorganisation of the actin cytoskeleton [6,7]. Two mammalian PLD genes have been identified, PLD1 and PLD2, each of which is expressed as two splice variants. Both isoforms have PX and PH domains and four conserved PLD signature domains. A catalytic HxKxxxxD triad is found within both domains I and IV with an additional GSANIN found in domain IV within which the S has been shown to be essential for catalysis [1]. Despite the linkage between receptor activation and the stimulation of PLD being recognised for more than 10 years, the mechanism is incompletely defined.

2. Activation by protein kinase C

PLD activation is frequently concomitant with the stimulation of phospholipase C activity in stimulated cells. A potential linkage between these two enzymes can be made since inhibition of protein kinase C, an enzyme downstream of phospholipase C, blocks the stimulation of PLD activity [8]. The mechanism of PLD activation by protein kinase C is however unclear. The effects of protein kinase C inhibitors suggested that PLD phosphorylation may be the mechanism of activation, however whilst detectable [9], phosphorylation in vitro is without significant effect upon activity. Furthermore, whilst the regulatory domain of protein kinase C has been shown to directly interact with PLD, this interaction is independent of kinase activity as the stimulation of PLD activity in vitro occurs in the absence of ATP. Consistent with this, a truncated protein kinase C devoid of the catalytic domain was found to be capable of activating PLD [10]. Mutation studies have now identified a region in PLD1 apparently N-terminal to the PX domain where protein kinase C interacts [11], this site thus forms one part of a triad of membrane attachment motifs together with the PH and PX domains (see below). This unique means of activation cannot however fully explain the mechanism of activation of PLD by protein kinase C, since kinase inhibitors suppress agonist-stimulated PLD activity in most cell types. The most likely explanation is that protein kinase C may be effecting the activation of other PLD regulators, or regulating the formation of a multi-component regulatory complex.

3. Activation by inositol phospholipids

In addition to the protein kinase C binding motif, the N-terminal region of both PLD1 and 2 contains the PX and PH domains. The PH domain of PLD is selective for bisphosphorylated inositides having a vicinal pair of phosphates, i.e. PtdIns(4,5)P_2 and PtdIns(3,4)P_2 [12]. PtdIns(3,4,5)P_3 is a weak activator of the purified enzyme, however it has a more significant activating effect in partly purified preparations. In surface plasmon resonance experiments there was only limited binding of PtdIns(3,4,5)P_3 to the intact phospholipase and none to the isolated PH domain, in contrast to the binding of PtdIns(4,5)P_2. Whilst the PH domain is clearly a major

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PtdIns(4,5)P_2 binding regulatory domain, other studies have provided evidence for an additional PtdIns(4,5)P_2 binding domain which is also apparently essential for activity [13]. Binding of PtdIns(4,5)P_2 to the PH domain of PLD has two functions, firstly mutation reduces or inhibits activation, but secondly complete removal of the domain both ablates activity and prevents membrane association [12]. Deletion of the N-terminus of PLD1, including the PX domain and the protein kinase C binding site as well as the PH domain, did not decrease PLD activity. Indeed the N-terminal deletion increased activity and did not prevent membrane association, however whether the mutated enzyme localised to and translocated between the same membranes as the wild type protein was not determined. It is therefore possible that deletion of this region affects in vivo regulation of the enzyme by localisation (see below).

The surface plasmon resonance studies showed that PLD also bound PtdIns(3)P, but that the binding was not within the PH domain [12]. Studies with VAM-7, p40 PHOX and SNX3 [14] have demonstrated that the PX domain binds PtdIns(3)P, thus it is probable that the PLD PX domain is also a PtdIns(3)P binding motif. As observed with the other PX domain containing proteins, the motif appears to be important in the endosomal localisation of PLD1 in particular. The direct evidence of lipid specificity and function of the PX domain in PLD remains to be provided, nevertheless there would appear to be a complex relationship between the PH and PX domains in PLD regulation by inositol phospholipids with an additional indirect effect upon the protein kinase C binding domain. This relationship is probably partly reflected in the control of PLD's intracellular localisation and therefore its interaction with its regulators (see below).

4. Small GTPases and PLD activation

Whilst PtdIns(3,4,5)P_3 does not bind to PLD and does not activate the enzyme in vitro, there is clear evidence from inhibitor studies for a role for PI-3-kinase activation in controlling PLD [15]. These results pointed to mechanisms of PLD activation following cell surface receptor occupation proceeding indirectly for example via PtdIns(3,4,5)P_3-regulated proteins. The demonstration that PLD activity in broken cells required GTP led to the identification of two families of small GTPases as regulators of PLD1, firstly the Arf family [16,17] and subsequently members of the Rho family of proteins (Rho, Rac, Cdc42) [18]. Both families were identified as direct activators and have each been shown by surface plasmon resonance to bind to PLD1a [19]. Whilst no Arf binding site has been identified, Rho family proteins appear to bind to PLD1 between amino acids 984 and 1000 in human and rat PLD1 [20] and protein kinase C appears to bind between amino acids 61 and 70. Other GTPases such as Ral have subsequently been proposed to regulate PLD activity, however the control by this protein is more complex since it appears to be cooperative with Arf and apparently doesn't require GTP loading of the Ral [21]. The importance of PI-3-kinase in this activation of Arf and Rho proteins is suggested by the identification of exchange factors such as Tiam-1 [22] and the cytohesins [23] whose activity is controlled by PtdIns(3,4,5)P_3-dependent PH domains.

The Rho and Arf families both comprise multiple members each having different functions, surprisingly each is capable of activating PLD1, with only small differences, in particular a reduced effect of Cdc42, between the abilities of the individual members of both these GTPase families [24,25]. Further, transfection experiments have shown that activated Rho, Rac and Cdc42 can each activate PLD1 [1]. Nevertheless, the distinct physiological functions of the GTPases raise questions about the specificity of PLD activation. Thus we addressed the interaction of PLD1 with wild type GTPases in the RBL-2H3 model mast cell under control and antigen-stimulated conditions. In this cell line we and others have previously demonstrated an important role for PLD in the regulation of antigen-stimulated secretion of the contents of the specialised secretory lysosomes [4,26]. Incubation of the cells with a primary, but not a secondary or tertiary alcohol inhibited stimulated secretion without inhibiting the translocation of PLD1, apparently associated with the vesicles to the plasma membrane. This suggested that PLD1 was activated at the plasma membrane in response to cellular stimulation.

Antigen stimulation of RBL-2H3 cells activates the syk tyrosine kinase, phospholipase Cγ and PI-3-kinase in addition to PLD, thus pathways involved in the control of a number of PLD regulators are also activated. We adopted a confocal microscopy approach to examine the association of PLD1 with potential regulatory molecules in the RBL-2H3 cells. Where possible we made use of suitable specific antibodies to detect the localisation of the endogenous proteins, or if necessary we examined the localisation of HA- or GFP-tagged transfected proteins, in the latter experiments care was taken to ensure the lowest possible level of expression of the exogenous proteins. In the resting cell PLD1b exhibited a punctate presumably vesicular localisation which corresponded to markers of the secretory lysosome, there was minimal colocalisation with any potential regulator. Following antigen stimulation, PLD1b translocated to the plasma membrane where it selectively colocalised with regulatory molecules, i.e. Arf6 but not Arf1, Rac1 but not RhoA or Cdc42, and protein kinase Cα [19]. Concomitant with this colocalisation PLD activity was stimulated.

These data raise questions concerning the previously reported activation of PLD by other Arf and Rho proteins, the explanation for this apparent contradiction may relate to the plethora of cellular functions assigned to PLD. PLD activity has been suggested to play an important role in the fragmentation and reassembly of the Golgi apparatus in rat liver with a pool of PLD1 being associated with Golgi membranes [27], indeed we have recently detected colocalisation of PLD1b and Arf1 in the Golgi of Cos1 cells [2]. A number of studies have suggested that PLD is found in the Golgi, though this has been disputed, see [28] for discussion. However, involvement of PLD in processes as distinct as GLUT4 glucose transporter translocation [29,30], EGF [5] and FcγRI receptor internalisation [31], hepatic very low density lipoprotein assembly [32] and the release of nascent secretory vesicles from the trans Golgi network [33] suggests that the enzyme plays a number of roles in trafficking events. These processes have also been variously suggested to involve Arf1, Arf6, RhoA and B and Rac1. There is also extensive evidence that PLD activation plays an important role in control of the actin cytoskeleton. A number of studies have shown that activation of PLD stimulates the formation of actin stress fibres in endothelial [7] and fibroblast cells [6], and using catalytically inactive constructs it has been demonstrated that

this may be a PLD1-mediated event [34]. Association of PLD with the actin cytoskeleton is further suggested by the importance of the Rho proteins and Arf6 in controlling actin rearrangements and in cell movement, for example a critical role for Arf6 and PLD activity in wound healing has recently been demonstrated [35]. Furthermore, actin has been shown to bind to PLD2 with an inhibitory effect upon phospholipase activity, and at least in vitro this is reversed by Arf1 [36]. However in vivo, a relationship between Arf6, rather than Arf1 and PLD2 together with PtdIns4-P-5-kinase Iα has been suggested [37].

The association between PLD2 and PtdIns4-P-5-kinase Iα has provided a mechanism for regulation of this PLD isoform. Whilst both PLD1 and PLD2 bind PtdIns4-P-5-kinase Iα, it is only the activity of PLD2 that is increased when the two are co-expressed; a catalytically inactive PtdIns4-P-5-kinase Iα also inhibits PLD2 activation [38]. Thus, it appears that PLD2 activity is regulated by PtdIns(4,5)P_2 availability. PtdIns4-P-5-kinase Iα activity is regulated by both PtdOH and Arf6, this provides a potential link between PLD1 and PLD2 activity and points to the cooperative roles of the two enzymes particularly in controlling cell shape change, an event in which PtdIns(4,5)P_2 is heavily implicated.

Whether each of these regulatory functions operates in the same cell under the activation of a single agonist is unknown, however in the RBL-2H3 model cell, antigen stimulates both the secretion of the contents of the secretory lysosome and the spreading and migration of the cell. This sequence of events operates in a number of stimulated haematopoietic cells, consequently there may be a coordinated regulation of the two PLDs in a number of cellular processes, the control of which will involve distinct small GTPases and inositol phospholipids.

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