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AN EVALUATION OF THE ROLE OF PHOSPHOLIPASE D ENZYMES IN BLOOD CELLS AND POSSIBLE IMPLICATIONS FOR HEALTH HOMEOSTASIS

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ABSTRACT

The enzyme Phospholipase D (PLD) is expressed in many blood cells like the erythrocytes, lymphocytes and platelets and may have critical roles in physiology and patho-physiology. The major substrate for the action of PLD is the Phosphatidylcholine. This enzymatic pathway induces the production of phosphatidic acid selectively. Phosphatidic acid is the precursor for diacylglycerol and lysophosphatidic acid. On the other hand, Guanine nucleotides also stimulate PLD enzyme in blood cells. Furthermore, tyrosine kinase may also be involved in platelet PLD regulation. It seems that multiple signals acting sequentially or in parallel converge on PLD enzyme. PLD is also present in platelet percursormegakaryocytric cells and can be activated by platelet agonists. In these cells both PKC and G-proteins (e.g. Rho) may regulate PLD activity. The significance of PLD in blood cells still requires thorough investigation. Recent research offer new avenues to further elucidate the biochemistry of this enzyme in blood cells. This review investigates the role of phospholipase D enzyme in lymphocytes and its implications in health homeostasis.

KEYWORDS: Phospholipase D, lymphocytes, platelets, erythrocytes, growth factors, enzyme.

INTRODUCTION

The phospholipase enzymes **(**PLDs) are signaling enzymes and are best known for their ability to generate phosphatidic acid (PA), an important lipid second messenger.[1] Among the cellular processes that are regulated by PLDs and their lipid products is vesicular trafficking, including endoplasmic reticulum-to-Golgi transport[2], post-Golgi secretion[3], and endocytosis. In blood cells like the lymphocytes, PLD has been found to regulate the exocytosis of CTLA-4-containing vesicles.[4] RhoA and Rac1 activation can leadto the generation of phosphatidic acid (PA), a signaling lipid that is produced by phospholipase D (PLD).[5] PLD generates PA through hydrolysis of phosphatidylcholine and this reaction additionally yields choline as a product.[6] PLD has also been described to be involved in processes of cell motion including platelet aggregation[7], lymphoma cell migration and neutrophil chemotaxis.[8] In the Lymphocytes two isoforms, PLD1 and PLD2 are expressed.[10] Previous studies suggested a functional role of PLD1 for regulating basic physiological cell functions such as degranulation, cytoskeletal reorganization, cell proliferation and migration.[11] Recently, it has been shown that PLD1 deficiency leads to impaired thrombus formation by altering αIIbβ3 integrin inside out signaling in platelets.[12] Furthermore, PLD1 activity has been implicated in the regulation of neutrophil chemotaxis, the motion and adhesion of leukocytes.[7,13] as well as lymphoma migration.[14] Recent studies also demonstrate that PLD1 is crucial for lymphocyte adhesion and transmigration through endothelial barriers under conditions of CNS autoimmunity.[15]

Lipid rafts and Phospholipase D activation

A decrease in lymphocyte responses associated with PLD activation has also been shown for the anti-proliferative cytokine IFN-β, which has been shown to decrease IL-2 production by Con A-activated PBMCs while inducing a sustained increase in phosphatidic acid level. The process of PLD1 activation inhibits lymphocyte proliferation remains speculative. It may be hypothesized that the molecular species of second messengers PA and DAG generated by PLD through PC hydrolysis have reduced signaling properties, as compared with the more unsaturated ones produced from PIP_2 hydrolysis.[16] PA may also have specific intracellular targets involved in the inhibition of the proliferative response. Among the different enzymatic targets of phosphatidic acid, the protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase 1 has been shown to bind PA with a high affinity, this interaction resulting in the stimulation of its phosphatase activity.[17] Thus, it can be assumed that in lymphocytic cells with elevated PLD activity, Src homology region 2 domain-containing phosphatase 1, which is well recognized as a negative regulator of T cell function, could be activated. Another interesting

hypothesis ensues from the recent observations that actin depolymerization must occur at the onset of immune response to allow the clustering of lipid rafts and the formation of the immune synapse.[18-20] Indeed, blocking actin depolymerization results in an inhibition of lymphocyte activation. As PLD activation is well known to induce actin polymerization, it may be supposed that increased PLD activity will counteract the initial depolymerization and then compromise cell response. Another possibility is that high PA level confers to lymphocytes in a preactivated state leading to loss of energy or apoptosis upon subsequent mitogenic activation.[21]

Phospholipase D and T lymphocytes

The two isozymes PLD1 and PLD2 are about 50% identical in sequence and display similar domain structures. But, they localize to different regions in the cell.In addition Jurkat T cellsalso express GTP-dependent PLD activity.[22] PLD protein and mRNA expression has been well demonstrated in several established lymphocytic cell lines, but only few reportshave addressed the PLD of human PBMC. A PLD activity, detected by means of the transphosphatidylation reaction, hasbeen described in these cells.[23] However, the identity ofthe PLD isoforms in presence is not precisely known. It is suggested that localization determines intermolecular interaction with downstream target molecules thus serving different functions in signal transduction.[24] A previous publication report shows that deletion of the loop region in PLD1 led to an increase in its basal activity levels but the localization of the loop deletion mutant was not determined. The expressed loop deletion mutant in the Jurkat T cell line and it was found that it had plasma membrane localization similar to PLD2. Since PLD2 is characterized by high basal activity it is quite possible that the increased activity of the loop deletion mutant is in part due to its plasma membrane localization and potential modification by membrane attached enzymes (eg: phosphorylation). An intriguing observation was that the isolated loop domain had plasma membrane localization.

Recent studies demonstrate that PLD2 enzyme is dispensable for development of T cells after the double positive stage of thymocyte differentiation. This does not however address the role of PLD2 in earlier aspects of T cell development. Also, it was found that deficiency of PLD2 did not affect CD4 T cell activation and proliferation. On the other hand, it was observed that differences in cytokine secretion profiles of PLD2 deficient T cells. PLD2 deficient T cell cultures had more IL-2 positive cells than wild type T cells and showed resistance to differentiate in to Th1 and Th2 lineage *in vitro*. This effect on effector T cell differentiation might because by blockade of transcription factors like T-bet and GATA3. These possibilities can be investigated in the future. Surprisingly, PLD2 deletion had less effect, if any, on the expansion of regulatory T cells. One possible explanation is that PLD1 compensates for the deficiency of PLD2. This hypothesis can be tested for this possibility by inducibly deleting PLD1 alone in PLD2 CD4 knock out mice. Another possibility is that PLD2 deficient T cells are inherently different due to their thymocyte development and differentiation in the absence of PLD2 signals. The other exciting possibility is perhaps that PLD2 is more important under conditions of physiological stress.[16] This possibility can be addressed by inducing autoimmunity or by observing the immunological response to pathogens in PLD2 deficient mice *in vivo*. Nonetheless, these experiments in PLD2 knock out mice have brought to light previously unknown queries.

Phospholipase D Assay in lymphocyte cells

Lymphocyte cells are harvested from P25 TCR transgenic mice and incubated overnight with (1-10ug/ml) P25 peptide. This is a earlier described modified technique.[25] The following day cells were harvested, washed with RPMI three times to remove any residual antigen peptide. CD4 T cells are sorted and kept at room temperature until further use. Cells are then suspended in 10% RPMI containing 10mM HEPES (1-$5x10^6$/500ul) and pretreated with DMSO or AB-MECA (50uM) for 30 mins in 37 degree water bath. Following pretreatment, CD4 T cells are stimulated with soluble biotin conjugated anti-CD3 and avidin for different time points (10-30 mins). Cells are centrifuged and resuspended in RIPA lysis buffer (10mM phosphate buffer pH 7.2,150mM NaCl, 1% Triton-X, 0.4% sodium deoxycholate, 0.1% SDS) containing Dnase. Cell lysates were assayed using the commercially available Amplex Red PLD assay kit (Invitrogen (Molecular Probes, USA). In this assay, PLD activity is monitored indirectly by using 10-acetyl-3, 7-dihydrophenoxasine, called the Amplex Red reagent. PLD cleaves the L-α-phosphatidylcholine (PC) substrate to yield choline and phosphatidic acid. Choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2 in the presence of horseradish peroxidase reacts with the Amplex Red reagent to generate the highly fluorescent product resorufin (excitation and emission maxima, ~568 and 589 nm, respectively), an H_2O_2 fluorogenicsonde. As per the manufacturers manual, this kit is specific for membrane PLD enzymes, with optimal activity at nearly neutral pH (pH 7.9). Purified PLD from Streptomyces chromofuscus and 10 µM H_2O_2served as positive controls. The PLD assay kit can detect PLD levels as low as 10 mU/ml. One unit of PLD is defined as the amount of enzyme that liberates 1.0 µmol of choline from PC per minute at pH 7.9 at 30°C. Cell lysates were incubated withthe reagents provided in the kit for 1hr at 37 degree and the fluorescence intensity was read using fluorometer (GA Instruments Pvt. Ltd., India).

Role of Phospholipase D enzyme in Arachidonic acid release

The released Arachidonic acid (AA) is metabolized to a variety of bioactive substances such as prostaglandins

(PGs). It has been reported that the major PG synthesized in vascular smooth muscle cells is PGI_2. In that study the extracellular ATP increased the synthesis of 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2, in rat aortic smooth muscle cells as has been shown by similar studies conducted on cardiovascular cells.[26-27] In addition, both propranolol and RHC-80267 suppressed ATP-induced 6-keto-$PGF_{1\alpha}$ synthesis as well as AA release in these cells. Thus, it is most likely that phosphatidylcholine hydrolysis by phospholipase D, which results in diacylglycerol formation, is involved in the extracellular ATP-induced synthesis of PGI_2 in rat aortic smooth muscle cells. In the lymphocytes the phospholipase D enzymes may be involved in the synthesis of prostaglandins by partially regulating the release of arachidonic acid as shown in Table 1.

Table 1: Effect of phospholipase D inhibitors (SCH 49209) and interleukin 3 on arachidonic acid release from Jurkat cells

Preatreatment	Treatment	[^{3}H]Arachidonic acid release (d.p.m)	% change from control values
1) Vehicle	IL3 (250u/ml)	2420	21.48
2) Vehicle	SCH49209 (2µg/ml)	1893	4.96
3) SCH 49209 (2µg/ml)	IL 3	2209	10.88
4) Vehicle	Vehicle	1992	--

Cannabinoids and influence on phospholipase D in blood cells ?

Whether the cannabinoids either exogenous or endogenous have a role in modulating the phospholipase D activity in blood cells like the lymphocytes has not been explored. It is well established by experimental evidence that endocannabinoids are synthesized by a mediated pathway. It is well known that Anandamide is formed from the one-step hydrolysis catalyzed by NAPE-PLD. However, since NAPE-PLD knockout mice display normal levels of anandamide in the brain and some other tissues[28], additional pathways for its formation must exist. Three additional pathways have been demonstrated that catalyze the formation of anandamide from N-arachidonoyl phosphatidylethanolamine. First, phospholipase C catalyzes an intermediate phospho-anandamide which is then dephosphorylated by a phosphatase[29]; secondly, the α/β-hydrolase 4 leads to the formation of a glycerol-phosphoanandamide which is then hydrolyzed further by phosphodiesteraseto form anandamide and third, it was shown that anandamide could be formed from the conversion of N-arachidonoyl phosphatidylethanolamine to a 2-lyso intermediate by soluble phospholipase A2 followed by cleavage by lyso-phospholipase D. The putative endocannabinoid membrane transporter has been characterized, but never cloned, and there is evidence for and against its existence.[30] There are a number of other putative endocannabinoids, including N-arachidonoyldopamine (NADA), an agonist at both CB1 and TRPV1 receptors[31] and virodhamine, a potential endogenous antagonist of the CB1 receptor.[31-33]

CONCLUSION

The recent information about the importance of phospholipase D enzyme in the blood cells is throwing more light on the importance of this enzyme in T cell mediated immunity and its role in cancer biology. Also important is to investigate the importance in cannabinoid mediated effects both in the gastrointestinal system and hematological system. Thus it appears that this enzyme has multifaceted role to play in both physiological and pathophysiological conditions. There is also growing impetus on the enzyme being a target of therapeutic agents.

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