Phospholipase A_2 (*EC* 3.1.1.4) and D (*EC* 3.1.4.4) signalling in lymphocytes

Michael J. O. Wakelam[1]* and Margaret M. Harnett[2]

[1]*Institute for Cancer Studies, Birmingham University Medical School, Birmingham B15 2TA, UK*
[2]*Department of Immunology, Glasgow University, Glasgow G12 8QQ, UK*

The stimulation of a whole range of cell types by cytokines, antigen receptor ligands, growth factors, or hormones effects changes in cellular responses through the activation of lipid signalling pathways. In many cases this entails the activation of phospholipases, in particular phosphatidylinositol 4,5-bisphosphate-phospholipase C (*EC* 3.1.4.11; PI-PLC), cytosolic phospholipase A_2 (*EC* 3.1.1.4; $cPLA_2$) and phospholipase D (*EC* 3.1.4.4; PLD). Activation of these enzymes generates signalling products which initiate intracellular signalling cascades. PI-PLC activity generates inositol 1,4,5-trisphosphate and *sn*-1,2-diacylglycerol (DAG), both of which can function as messengers. Inositol 1,4,5-trisphosphate stimulates the release of Ca^{2+} from intracellular stores and DAG is the physiological activator of protein kinase C (PKC). $cPLA_2$ catalyses the hydrolysis of phosphatidylcholine (PC) generating lysophosphatidylcholine and arachidonate (AA) as products. PLD also utilizes PC as a substrate; however, its activation generates free choline and phosphatidate (PA) as its lipid product (Wakelam, 1994).

AA and PA have both been proposed to have messenger properties. AA is the precursor lipid for eicosanoid biosynthesis, but has also been shown to be a co-activator of certain PKC isoforms, and to have ill-defined effects on a number of small-molecular-weight GTP-binding proteins. In addition, AA has been suggested to have effects on transcription mediated by the transcription factor AP-1 (Kim & Kim, 1997). PA activates a partly-purified protein kinase in neutrophils which is an activator of the NADPH oxidase (Waite *et al.* 1997). PA also has been shown to mediate Rho-dependent actin stress fibre formation in porcine aortic endothelial cells, and plays a stimulatory role in secretion in neutrophils and mast cells (Cross *et al.* 1996). PA is dephosphorylated by PA phosphohydrolase (*EC* 3.1.3.4) activity to generate DAG and this has been proposed to result in sustained PKC activation.

The activation of PI-PLC in lymphocytes, in particular the γ isoform, has been extensively reported (Pao *et al.* 1997; Taylor-Fishwick *et al.* 1997). However, few studies in the literature have described the regulation of PLD or $cPLA_2$ activity in B or T lymphocytes. Nevertheless, activation of both enzymes has been observed and been proposed to be involved in both proliferative and apoptotic responses (Stewart *et al.* 1991, 1993; Gilbert *et al.* 1993, 1996; Mollinedo *et al.* 1994; Reid *et al.* 1997; JJ Gilbert, MJO Wakelam and MM Harnett, unpublished results). The present review discusses the functions of the lipid messengers generated by PLD and $cPLA_2$ activation, and further considers the regulation of these lipid signalling pathways in lymphocytes.

Phospholipase D

PLD is the product of a multi-gene family of which PLD1 and 2 have been cloned (Wakelam *et al.* 1997). PLD activity shows an absolute requirement for phosphatidylinositol 4,5-bisphosphate, and both isoforms can be regulated by PKC. However, PLD1 is more potently activated, and this isoform is also regulated by the small GTP-binding proteins Arf, Rho and Ral. The regulation of PLD2 is less-well defined. The PA product of PLD activation is probably a direct messenger molecule. Hydrolysis of PA by PA phosphohydrolase generates DAG which has been proposed to activate PKC. However, activation of PKC can stimulate PLD activity, and thus the potential for unregulated signalling exists. This clearly does not occur; indeed agonist-stimulated PLD activation has been reported to be desensitized in many cell types, calling into question either the physiological importance of PLD regulation by PKC, or the ability of PLD-generated DAG to activate PKC.

Molecular-species analysis has demonstrated that cells contain upwards of fifty distinct DAG, distinguishable by acyl chain structure. Stimulation of many cell types induces a biphasic increase in DAG mass, of which the increased primary phase is predominantly represented by DAG with a polyunsaturated fatty acid at the *sn*-2 position (Pettitt & Wakelam, 1993). This initial accumulation of polyunsaturated DAG is generally limited to a few molecular species, notably 1-stearoyl,2-arachidonyl-DAG, an acyl chain combination that is abundant in inositol phospholipids. The second, more sustained phase of DAG generation is predominantly made up of more saturated species. Analysis of the acyl chain structure of cellular phospholipids suggests that the initial DAG phase is indeed derived from

Abbreviations: AA, arachidonate; BCR, B-cell antigen receptor; $cPLA_2$, cytosolic phospholipase A_2; DAG, diacylglycerol; Ig, immunoglobulin; PA, phosphatidate; PC, phosphatidylcholine; PI-PLC, phosphatidylinositol 4,5-bisphosphate-phospholipase C; PKC, protein kinase C; PLD, phospholipase D; TCR, T-cell antigen receptor.

***Corresponding author:** Professor Michael J. O. Wakelam, fax +44 (0)121 414 5263, email M.J.O.Wakelam@bham.ac.uk

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phosphatidylinositol 4,5-bisphosphate hydrolysis, whilst the sustained phase is more representative of PC. The typical DAG species generated in stimulated fibroblasts (Swiss 3T3) and porcine aortic endothelial cells are outlined in Table 1.

Despite the PC pool being predominantly saturated or monounsaturated, many cells also have a minor pool of PC which is polyunsaturated. Thus, some of the primary phase of polyunsaturated DAG could be PC-derived if a PC-phospholipase C activity was operating, as has been suggested for epidermal growth factor-stimulated Swiss 3T3 cells (Pettitt *et al.* 1994). We have also recently observed that polyunsaturated DAG, which appear to be the result of PC-phospholipase C activity, are generated in $CD4^+$ T-cells. The selective generation of polyunsaturated DAG by phospholipase C activation implies an acyl chain substrate specificity of the phospholipase in intact cells which has not been detected in *in vitro* experiments. Alternatively, there may be specific pools of particular species in particular intracellular locations corresponding to the point of activation of the individual phospholipase.

The lipid-species analysis also suggests that the more polyunsaturated DAG are the physiologically-relevant messenger molecules. If this supposition is correct, then these DAG should be generated when PKC is being activated in cells. In Swiss 3T3 cells this appears to be the case. Bombesin stimulates a transient generation of polyunsaturated DAG, a transient activation of PKC and is non-mitogenic. Bombesin together with transforming growth factor β is mitogenic for Swiss 3T3 cells, it also stimulates the sustained generation of a number of polyunsaturated DAG and the sustained activation of PKC (Olivier *et al.* 1996). In contrast, in porcine aortic epithelial cells lysophosphatidic acid only stimulates an increase in the more saturated DAG and does not appear to activate PKC (Pettitt *et al.* 1997).

PLD activation can be monitored in cells by determining the generation of phosphatidylalcohols. The PLD reaction is a transphosphatidylation in which water displaces the enzyme from an apparent phosphatidyl-PLD intermediate (Wakelam *et al.* 1997). In the presence of a short-chain primary aliphatic alcohol, e.g. ethanol or butanol, the reaction preferentially utilizes the alcohol as a nucleophilic acceptor and generates a phosphatidylalcohol. Phosphatidylalcohols appear to be poor substrates for PA phosphohydrolase and other metabolizing enzymes, and thus accumulate in cells. Use of this characteristic provides unequivocal evidence that a particular lipid species has been generated through the action of a PLD. Thus, species analysis of a phosphatidylalcohol is equivalent to a selective analysis of the acyl structure of that portion of the cell's PA which has been generated by PLD. Such an analysis has only been performed in a few studies; however, in each case a greater proportion of less-saturated lipids has been generated compared with the more polyunsaturated species found in the phospholipase C-derived DAG (Pettitt *et al.* 1997). The DAG generated from these PA will therefore also be predominantly saturated and monounsaturated. These findings strongly suggest that the 'signalling PA' is saturated or monounsaturated acyl chains and thus that polyunsaturated PA, like the more saturated DAG, are non-signalling metabolites. If this is the case, then there will probably not be the signalling cross-talk suggested previously, since the DAG generated through the PLD pathway will not significantly activate PKC.

Table 1. Major diacylglycerol (DAG) species changes in bombesin-stimulated Swiss 3T3 and lysophosphatidic acid (LPA)-stimulated porcine aortic endothelial (PAE) cells (Data from Pettitt & Wakelam (1993) and Pettitt *et al.* (1997))

Stimulated DAG species	Cell type
1° 18 : 0, 20 : 3*n*-9; 18 : 0, 20 : 4*n*-6; 18 : 0, 20 : 5*n*-3	
2° 18 : 1*n*-9, 18 : 1*n*-9; 16 : 0, 16 : 0; 16 : 0, 18 : 1*n*-9; 18 : 0, 18 : 1*n*-9; 16 : 0, 18 : 0	
Basal* has elevated 18 : 0, 20 : 4*n*-6 and 18 : 0, 20 : 3*n*-9	Swiss 3T3 PAE
Increase in 18 : 1*n*-9, 18 : 1*n*-9; 16 : 0, 16 : 1; 14 : 0, 18 : 1*n*-9†	

1°, 2°, primary and secondary phases respectively of increased DAG.
* Resting level; this is in comparison with other cell types.
† LPA-stimulated changes.

Differential roles for phospholipase D in B- and T-cell signalling

As described previously, PC-PLD has been proposed to play a key role in the transduction of the proliferative responses of a wide range of mitogens and growth factors. Interestingly, the T-cell antigen receptor (TCR) has recently been shown to be coupled to PLD activation in the leukaemic T-cell line, Jurkat, resulting in the induction of the transcription factor AP-1, a signalling event involved in the regulation of growth and proliferation of T-cells (Stewart *et al.* 1991, 1993; Mollinedo *et al.* 1994). Consistent with this finding, we have found that the antigen receptors on mature T lymphocytes derived from human tonsillar or murine splenic preparations are also coupled to PC-PLD activation following stimulation of these T cells with anti-CD3 antibodies or the mitogenic lectins, phytohaemagglutinin or concanavalin A (Reid *et al.* 1997). However, as we have also demonstrated that the antigen receptors on murine thymocytes are coupled to PC-PLD activation in cells that are programmed to undergo cell death by apoptosis (Reid *et al.* 1997), it is unlikely that this PLD pathway plays a central role in the transduction of T-cell proliferative responses. It is likely that it is involved in either driving cells into cycle or maintaining cell cycle progression, processes required both for proliferation and activation-induced cell death. Whilst the molecular mechanisms underlying TCR coupling to PC-PLD activation in these cells have not been fully defined, kinetic studies and experiments using pharmacological inhibitors of protein tyrosine phosphatases and reconstituting CD3-coupled PC-PLD activity in streptolysin-O permeabilized cells suggest that the TCR–CD3 complex, under optimal conditions of activation, may be predominantly coupled to PC-PLD activation downstream of tyrosine phosphorylation of phospholipase C-γ, phosphatidylinositol 4,5-bisphosphate hydrolysis, Ca mobilization and PKC activation (Reid *et al.* 1997).

In contrast, in B lymphocytes, the Ca-mobilizing antigen receptors are not coupled to PC-PLD following stimulation with mitogenic anti-immunoglobulin (Ig) antibodies (Gilbert *et al.* 1993; JJ Gilbert, MJO Wakelam and MM Harnett,

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unpublished results). In addition, PC-PLD is not stimulated following cross-talk between surface Ig antigen receptor, interleukin-4 receptors, major histocompatibility complex class II molecules and other T-cell-interacting B-cell co-receptors such as CD40, conditions designed to mimic T-cell-dependent B-cell activation. However, ATP, which inhibits surface Ig antigen receptor-mediated DNA synthesis in murine B-cells via P_2-purinoceptors, does activate PC-PLD (Gilbert *et al.* 1993; JJ Gilbert, MJO Wakelam and MM Harnett, unpublished results). Moreover, ceramide and sphingosine, which also exert anti-proliferative effects on B-cells, also stimulate PC-PLD. Thus, whilst PC-PLD is associated with the transduction of anti-proliferative signals in mature B-cells, it does not transduce early signals associated with surface Ig-mediated growth arrest in immature B-cells (Gilbert *et al.* 1993, 1996). Mitogenic stimulation of surface Ig is, however, coupled to a novel non-PC-hydrolysing PLD activity. The resultant surface Ig-generated PA, unlike the PC-derived PA generated via the purinoceptors, is converted to DAG. These findings suggest that whilst the novel surface Ig-coupled PLD and resultant DAG generation may play a role in B-cell proliferation, PLD may transduce, via PA, negative immunomodulatory signals in mature B lymphocytes (Gilbert *et al.* 1993; JJ Gilbert, MJO Wakelam and MM Harnett, unpublished results).

Phospholipase A_2

$cPLA_2$ is the major intracellular PLA_2 activated by receptor stimulation. This 85 kDa enzyme appears to be regulated by phosphorylation on ser505, probably catalysed by mitogen-activated protein kinase, and an increase in intracellular Ca^{2+} concentration. It exhibits a specificity for a polyunsaturated fatty acid at the *sn*-2 position, generally releasing AA and, whilst not exhibiting any specificity *in vitro*, preferentially hydrolyses PC in intact cells (Wakelam, 1994). The best characterized role for AA is as a precursor for the biosynthesis of eicosanoids; however, it has been suggested that the lipid itself can have signalling roles, e.g. as a co-activator of certain PKC isoforms, a regulator of Ca^{2+} concentration and, in an ill-defined manner, as an activator of neutral sphingomyelinase (*EC* 3.1.4.12) activity (Jayadev *et al.* 1994).

Differential expression and activation of cystolic phospholipase A_2 during B and T lymphocyte development

The molecular mechanism by which the antigen receptors on B- or T-cells can elicit differential maturation state-specific biological responses is one of the central problems in lymphocyte differentiation yet to be resolved. The immune system has evolved clonal selection mechanisms to prevent the maturation of B- or T-cells of autoreactive specificity. Thus, whereas ligation of the antigen receptors (surface Ig–B-cell antigen receptor (BCR)) leads to cellular anergy and/or death by apoptosis of immature (bone marrow) B-cells, so as to prevent emergence of autoreactive B-cells in the periphery, it induces proliferation and differentiation of mature B-cells into antibody- secreting cells or memory (germinal centre) B-cells. Moreover, in the germinal centres, B-cells, which have undergone somatic mutation resulting in a modified, and hence potentially autoreactive, antibody specificity, are programmed to die (peripheral tolerance) unless rescued by antigen and cognate follicular dendritic cell or T-cell-derived signals. Similarly, during T-cell development in the thymus, ligation of the antigen receptors (TCR–CD3 complex) on immature thymocytes ($CD4^+$ $CD8^+$) leads to cell death, whereas ligation of the TCR on mature T-cells results in proliferation, given appropriate co-stimulatory signals. Differential coupling of the antigen receptors to signalling pathways, in a maturation state-dependent manner, could clearly provide a molecular mechanism for the distinct biological responses observed following ligation of the BCR or TCR on immature and mature B- or T-cells. However, the earliest signalling events detected following antigen receptor ligation have proved to be similar in both immature and mature B- and T-cells (Pao *et al.* 1997; Taylor-Fishwick *et al.* 1997). In contrast, we have recently shown that, whilst the BCR on immature B-cells is coupled to the $cPLA_2$-mediated PC hydrolysis, the BCR on mature B-cells is not coupled to this signalling pathway (Courtney *et al.* 1993; Gilbert *et al.* 1996). Indeed, whilst $cPLA_2$ is strongly expressed in immature B-cells it cannot be immunodetected in precursor B-cells or mature B-cells (Courtney *et al.* 1993; Gilbert *et al.* 1996). Moreover, $cPLA_2$ expression is up-regulated in germinal- centre B-cells, which will also apoptose following ligation of the BCR unless rescued by additional signals transduced via receptors such as CD23, CD38 or CD40. Similarly, during T-cell development, we find that whilst $cPLA_2$ is expressed and coupled to the TCR–CD3 complex in $CD4^+$ $CD8^+$ double positive thymocytes which are subject to negative selection, it is neither activated through the TCR, nor expressed in naive mature splenic T-cells (Courtney *et al.* 1993; Gilbert *et al.* 1996). Furthermore, we find that $cPLA_2$ expression is up-regulated in mature T-cells primed for activation-induced cell death. In addition, the antigen receptors on such B- and T-cells are only coupled to $cPLA_2$ activation under conditions which induce cell death. Coupling to $cPLA_2$ activation under these conditions precedes sphingomyelinase-catalysed ceramide generation, which is implicated in the transduction of apoptotic signals. Taken together with the increasing evidence that $cPLA_2$ activity is suppressed during mitosis (Berlin & Preston, 1995) and that AA can mediate anti-proliferative or apoptotic signals in a number of cell types (Neale *et al.* 1988; Jayadev *et al.* 1994; VoelkelJohnson *et al.* 1996), the correlation of $cPLA_2$ expression and coupling of the BCR or TCR to AA generation in B- or T-cells at maturation stages committed to apoptosis, suggests that $cPLA_2$ activation could be deleterious for lymphocyte survival, selection and proliferation. Thus, restriction of $cPLA_2$ expression in lymphocytes in a maturation-dependent manner may provide a novel mechanism for controlling deletion of autoreactive B- and T-cells.

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