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Short Communication

# Reduction of phospholipase D activity during coxsackievirus infection

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During enterovirus infection, host cell membranes are rigorously rearranged and modified. One ubiquitously expressed lipid-modifying enzyme that might contribute to these alterations is phospholipase D (PLD). Here, we investigated PLD activity in coxsackievirus-infected cells. We show that PLD activity is not required for efficient coxsackievirus RNA replication. Instead, PLD activity rapidly decreased upon infection and upon ectopic expression of the viral 3A protein, which inhibits the PLD activator ADP-ribosylation factor 1. However, similar decreases were observed during infection with coxsackieviruses carrying defective mutant 3A proteins. Possible causes for the reduction of PLD activity and the biological consequences are discussed.

During enterovirus infection, extensive rearrangements and alterations of host cell membranes occur in a relatively short time, and several viral proteins bring about these alterations. For example, vesicular transport through the secretory pathway is inhibited during enterovirus infection (Doedens & Kirkegaard, 1995; Wessels *et al.*, 2005), membrane permeability increases (Aldabe & Carrasco, 1995) and massive vesiculation occurs (Aldabe & Carrasco, 1995; Bienz *et al.*, 1987; Cho *et al.*, 1994). Enteroviral replication takes place on these membrane vesicles; in fact, all positive-stranded RNA viruses replicate their RNA genome on vesicles, though their cellular origin may differ between viruses (Mackenzie, 2005).

In enterovirus-infected cells, the phospholipids that compose organelle and vesicle membranes are extensively altered. Besides a significant increase in *de novo* phospholipid synthesis that may result in changes in membrane lipid composition (Guinea & Carrasco, 1990), several cellular lipid-modifying enzymes may also contribute to these alterations. For example, activities of phosphatidylcholine-specific phospholipase C and phosphatidylinositol-specific phospholipase C increase, while phospholipase $A_2$ activity ($PLA_2$) decreases (Guinea *et al.*, 1989; Irurzun *et al.*, 1993). In these studies, phospholipase D (PLD) activity was not investigated. PLD is a ubiquitous lipid-modifying enzyme that catalyses the conversion of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) to phosphatidic acid (PtdOH) (Fig. 1a), and acts in various cellular signalling pathways, protein secretion and membrane trafficking (Exton, 2000; Ktistakis *et al.*, 2003). Alteration of its activity might contribute significantly to the lipid modifications that occur during infection. Hence, we set out to investigate the role and regulation of PLD activity during coxsackievirus B3 (CVB3) infection.

First, we determined whether PLD activity is required for coxsackievirus RNA replication. To this end, we transfected Buffalo green monkey (BGM) kidney cells with a subgenomic replicon of wild type (wt) CVB3, containing a firefly luciferase gene in place of the capsid-coding region; luciferase production from this replicon reflects viral RNA replication. Directly after transfection, cells were chronically treated with the commonly used PLD inhibitor, 1-butanol (Chalifa *et al.*, 1990). In the presence of a primary alcohol like 1-butanol, PLD transfers the phosphatidyl group of PtdCho and PtdEtn to the alcohol, generating phosphatidylalcohol rather than its normal product PtdOH. Measurement of luciferase activity at 8 h post-transfection showed that neither 0.4 % 1-butanol nor 2-butanol (which does not inhibit PLD and serves as a control) reduced viral RNA replication, whereas viral RNA replication was completely abolished by guanidine hydrochloride (GuHCl), a commonly used inhibitor of enteroviral RNA replication (Fig. 1b). These data suggest that PLD activity is not required for viral RNA replication.

Although PLD activity appeared to be dispensable for viral replication, it might be altered during enterovirus infection. To investigate this, we labelled cells metabolically overnight with [$^3$H]myristic acid, which is incorporated in all *de novo* formed phospholipids. Cells were then either

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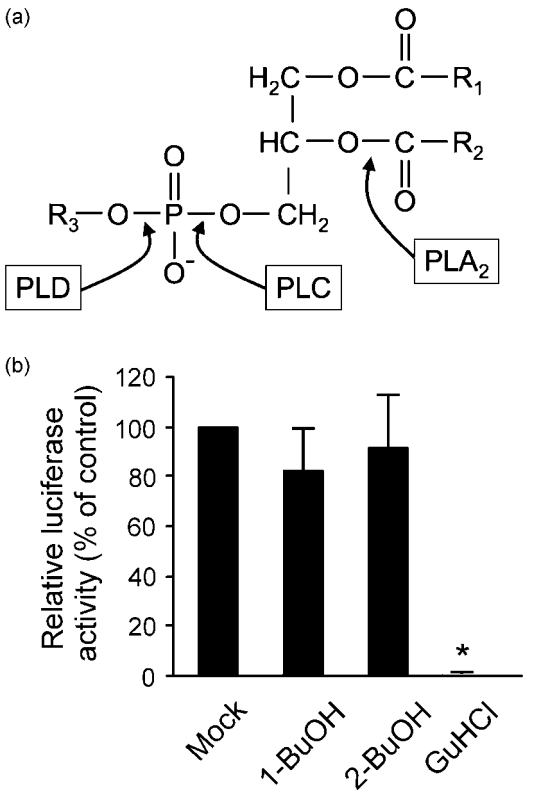


**Fig. 1.** Effect of PLD inhibition on enterovirus replication. (a) General structure of a phospholipid. $R_1$ and $R_2$ are fatty acid side chains, whereas $R_3$ is e.g. the choline or ethanolamine headgroup. The bonds hydrolysed by $PLA_2$, PLC and PLD are indicated. (b) BGM cells were transfected with a subgenomic luciferase replicon of CVB3, after which they were either mock treated or treated with 0.4 % 1-butanol, 0.4 % 2-butanol or 2 mM GuHCl. Luciferase activities were determined at 8 h post-transfection and compared to the activity in mock-treated cells, which was set at 100 %. Mean ± SEM of four independent experiments is shown. Differences were considered statistically significant (*) at $P<0.05$ (as calculated by Students *t*-test).

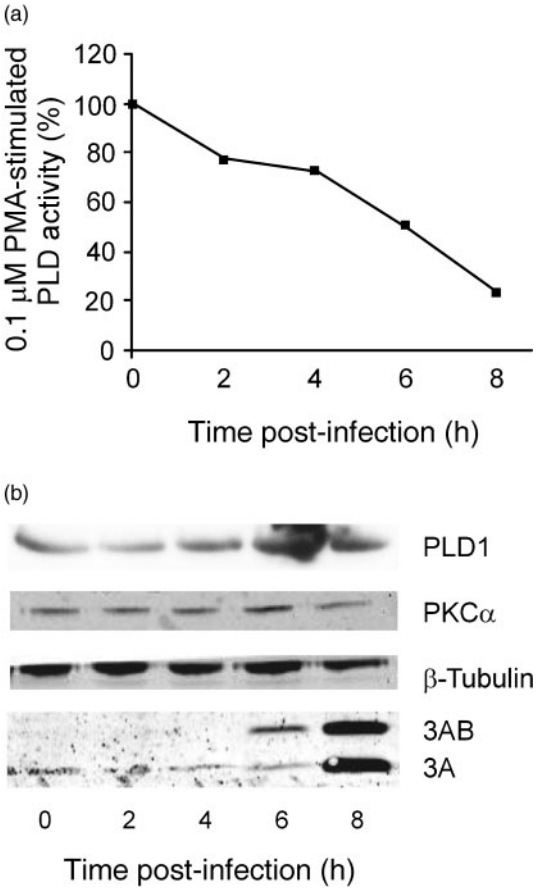


**Fig. 2.** Coxsackievirus infection reduces PMA-stimulated PLD activity. (a) BGM cells were either mock or CVB3 infected (m.o.i. of 50). Cells were harvested at 2, 4, 6 and 8 h p.i. and the 0.1 μM PMA-stimulated PLD activity was determined. PLD activity is expressed as a percentage of that in mock-infected control cells. One representative of two experiments is shown. (b) Expression levels of PLD1, PKCα, β-tubulin (loading control) and the CVB3 3A protein (and its precursor 3AB) during infection were measured by Western immunoanalysis. Antisera used were: (i) rabbit polyclonal antiserum against human PLD1 (Müller-Wieprecht *et al.*, 1998: kindly provided by Dr C. Geilen, Free University of Berlin, Germany); (ii) rabbit polyclonal antibody against PKCα (Cell Signalling Technology); (iii) mouse monoclonal antibody against β-tubulin (Sigma); (iv) rabbit polyclonal antiserum against CVB3 3A (Wessels *et al.*, 2006).

mock-infected or infected with CVB3 at an m.o.i. of 50 and harvested at 0, 2, 4, 6 and 8 h post-infection (p.i.). At 1 h prior to harvesting, ethanol was added to the medium to a final concentration of 1 %. Using this approach, PLD activity was directly reflected by the amount of phosphatidylethanol (PtdEth) produced. Subsequently, lipids were extracted as described previously (Bligh & Dyer, 1959), separated by TLC, and the amount of [$^3$H]PtdEth produced from the total [$^3$H]-labelled phospholipid pool was determined as described previously (Bosch *et al.*, 1999). Because of the low basal activity of PLD, we applied a short-term (1 h) treatment of the cells with phorbol-12-myristate-13-acetate (PMA), which greatly potentiates PLD activation (Chen & Exton, 2004; Lee *et al.*, 1997; Whatmore *et al.*, 1994). The results showed that PLD activity decreased as early as 2 h p.i., and continued to decrease down to 20 % of the level of mock-infected cells by 8 h p.i. (Fig. 2a). This decrease in PLD activity was, most likely, not due to reduced protein levels or proteolytic degradation of PLD, nor of its activator protein kinase C alpha (PKCα), as only minor decreases in PLD1 and PKCα protein levels were seen at these time points and no cleavage products were observed (Fig. 2b).

The small GTPase ADP-ribosylation factor 1 (Arf1) is a potent regulator of PLD activity (Brown *et al.*, 1993). We recently showed that the enteroviral 3A protein inhibits the

activity of Arf1 (Wessels *et al.*, 2006), and thereby it might also indirectly downregulate PLD activity. To test this, we transfected BGM cells with expression plasmids for enhanced green fluorescent protein (EGFP)-3A (Wessels *et al.*, 2005) or EGFP (Clontech) as a control. At 16 h post-transfection, cells were detached and viable, EGFP-positive cells were high-speed sorted by FACS using an Altra Hypersort flowcytometer (Beckman Coulter). EGFP-positive cells ($2 \times 10^5$) were collected, seeded and labelled with 2 μCi (74 kBq) [$^3$H]myristic acid. Subsequently, PLD activity was determined as described above. PLD activity was reduced to ~50 % in cells expressing 3A protein (Fig. 3a). We also tested the effects of mutant 3A proteins (EGFP-3A-P17A/P18A/P19A and EGFP-3A-P19A) that are unable to inhibit Arf1, as indicated by their inability to inhibit protein transport (Wessels *et al.*, 2005), on PLD activity. Upon expression of these mutants, PLD activity was not reduced. In contrast, expression of a mutant 3A protein, which was still able to inhibit Arf1 (EGFP-3A-P18A), caused a reduction in PLD activity similar to that caused by wt 3A protein (Fig. 3a). The FACS analysis showed that the wt and mutant EGFP-3A proteins were expressed to similar levels (data not shown), arguing that it is unlikely that the results are caused by differences in expression level.

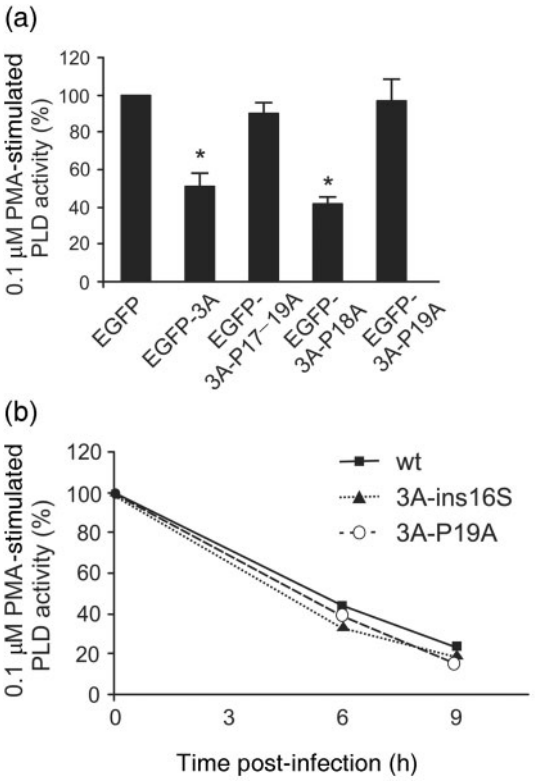


**Fig. 3.** Transient expression of 3A decreases PLD activity, but this function is not responsible for the virus-induced decrease in PLD activity. (a) Effect of 3A proteins on PLD activity. BGM cells were transfected with expression plasmids for EGFP, EGFP-3A, EGFP-3A-P17–19A, EGFP-3A-P18A or EGFP-3A-P19A. PLD activity in all treatments was compared to that in EGFP-expressing control cells, which was set at 100 %. *, $P<0.05$. (b) Effect of viruses carrying defective 3A proteins on PLD activity. BGM cells were either mock infected or infected with CVB3 wt, CVB3-3A-P19A or CVB3-3A-ins16S (m.o.i. of 50). Infected cells were harvested at either 6 or 9 h p.i. and the PLD activity was determined.

To determine whether the reduced PLD activity in CVB3-infected cells was caused by 3A-mediated inhibition of Arf1 activity, we determined PLD activity in cells infected with a virus carrying a mutant 3A protein defective in this function (CVB3-3A-P19A) (Wessels *et al.*, 2005). Upon infection with this virus, a decrease in PLD activity was observed similar to that caused by wt CVB3 (Fig. 3b). Also during infection with another virus carrying a defective 3A protein (CVB3-3A-ins16S) (Wessels *et al.*, 2006), PLD activity was strongly decreased (Fig. 3b). These findings suggest that the reduction in PLD activity in CVB3-infected cells does not correlate with 3A-mediated Arf1 inhibition.

What then may be the cause of the reduced PLD activity? No reduction in PLD or PKCα protein levels was seen, ruling out the option that decreased PLD activity is the result of inhibition of gene expression or degradation of these proteins. Our results show that the reduction in PLD activity is not caused by inhibition of Arf1 by the 3A protein. This finding, together with the observation that the level of active Arf1 increases during poliovirus infection (Belov *et al.*, 2007), strongly suggests that enteroviruses inhibit PLD activity at a step downstream of Arf1. Long-term disassembly of the Golgi complex, rather than Arf1 inhibition per se, has been suggested to be the underlying cause of decreased PLD activity (Guillemain & Exton, 1997). Enteroviruses cause a profound membrane reorganization, leading to the accumulation of vesicles at which viral RNA replication takes place, and, ultimately, the disassembly of the Golgi complex (Cho *et al.*, 1994). The viral 2BC protein has been shown to play a major part in this membrane reorganization (Cho *et al.*, 1994) and thereby may affect PLD activity. In our hands, however, ectopic expression of 2BC protein was rather inefficient and therefore we could not test the possible effects of 2BC protein on PLD activity. To date no viable mutant viruses have been described expressing a defective 2BC protein because of the important role of 2BC protein in viral RNA replication. For these reasons, we were unable to determine the involvement of 2BC protein in the downregulation of PLD activity. Although 2BC-mediated effects on the Golgi complex may offer a plausible explanation for the decrease of PLD activity in infected cells, other explanations, which may be non-mutually exclusive, cannot be ruled out.

What would be the consequence of a reduced PLD activity for the virus? PLD is a ubiquitous enzyme that generates PtdOH, an important second messenger (Exton, 2000). PtdOH in turn can be used by phosphatidic acid phosphohydrolase and diacylglycerol lipase to generate

arachidonic acid (AA) (Serhan *et al.*, 1996), a very potent second messenger that can be converted to several eicosanoid hormones, like prostaglandins, which play important roles in sustaining inflammatory responses and display antiviral activities against picornaviruses (Ankel *et al.*, 1985; Conti *et al.*, 1996, 1999). Besides PLD, $PLA_2$ can also generate AA directly from membrane phospholipids (Fujita *et al.*, 1996), and it was shown that $PLA_2$ is strongly inhibited during poliovirus infection, severely reducing the amount of AA produced (Guinea *et al.*, 1989). By additionally interfering with PLD activity, the virus would reduce even further the production of AA, thus helping it to evade the immune system.

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