Plant phosphatidic acid metabolism in response to environmental stress

Arisz, S.A.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
General discussion
General discussion

In *Chlamydomonas* as well as seed plants such as *Arabidopsis*, the induced accumulation of PA, a known lipid second messenger (LSM) in mammalian systems, has emerged as a common element in the acute phase of various plant stress responses. Determining the biochemical pathways underlying this PA response is the main research objective of this thesis.

In the web of lipid metabolism, PA takes a central position (Chapter 1). It is precursor in de novo biosynthesis of glycerolipids, which includes phospholipids, galactolipids, sulfolipids, and triglycerides. As such it is formed by successive acylations of the two free hydroxyls of glycerol 3-phosphate. For the synthesis of PE, PC, and galactolipids, this nascent PA is dephosphorylated to DAG, whereas for the synthesis of PI, and PG it is converted to CMP-PA. DAG and CMP-PA are substrates in reactions by which they receive their polar headgroups (Chapter 1).

Alternatively, PA can be formed through the inducible activities of PLD, hydrolyzing a structural phospholipid (e.g. PE or PC), or by DGK, which phosphorylates DAG. The DAG substrate for DGK can be provided by PLC, which hydrolyzes PPIs, such as PI4P, or PI(4,5)P2. Thus, PA originates from multiple cellular subpools, and distinguishing them is crucial to understanding the nature of the observed increases of this lipid under stress conditions. PA-increases measured in total lipid extracts, however, do not tell which metabolic route was responsible. To discriminate between PAs resulting from different pathways, several approaches were used. The first approach we applied makes use of differential in vivo 32P-radiolabelling ([1, 2], Chapter 2). When cells are incubated with 32Pi, label incorporation into ATP and, consequently, labelling of the products of ATP-dependent phosphorylation such as DGK-derived PA, is fast (within minutes). In contrast, labelling of PA derived from PLD activity takes much longer labelling times (hours-days) reflecting the slow labelling kinetics of PLD’s substrates, e.g. PE or PC. Hence, short labelling conditions monitor DGK-generated PA, and long labelling times predominantly show PLD-derived PA. To specifically probe PLD, its unique ability to catalyze a transphosphatidylation reaction is used. In the presence of an alcohol such as butanol, the phosphatidyl moiety of a structural phospholipid is transferred to the alcohol, generating PBut, which can be visualized by TLC separation and is used as an in vivo marker of PLD activity [3].

Finally, we applied a method relying on fatty acid compositions of phospholipids as distinguishing features of the cellular subpools. These features are passed on to downstream metabolic products, raising the possibility to directly link precursors to products, and vice versa. Thus, during stress signalling, the fatty acid composition of PA reflects not only de novo synthesized species, but also the composition of PPIs when a PLC-DGK route is active, or of a structural phospholipid when PLD is involved.

To apply this method in *Chlamydomonas*, it was necessary to establish the fatty acid compositions of its glycerolipid classes, including the PPI and PA, by gas chromatography.
Subsequently, to determine which phospholipid served as substrate to PLD during stimulation with various stresses, the fatty acid and molecular species composition of PBut was analyzed. Irrespective of the stimulus, di-oleoyl species prevailed, matching the predominant species in PE, identifying the latter as the PLD substrate ([5], Chapter 4). Analysis of NaCl-stimulated PA showed a mixture of PPI- and PE-like molecular species, suggesting the involvement of both PLC-DGK and PLD pathways. In principle, as discussed in Chapter 1 and 7, the activity of inositolphosphoceramide synthase (IPCS) would also be able to generate DAG at the expense of PPI, however this is not confirmed by the $^{32}$P-radiolabelling patterns during salt stress. The suggested formation of PLC/DGK- and PLD-generated PA is in agreement with previous results indicating the involvement of both pathways in the salt stress response ([6]). Based on fatty acid analysis of *Chlamydomonas* cells treated for 5 min. with 150 mM NaCl, we concluded that the PLD contribution to PA was dominant.

In contrast, in the response of *Arabidopsis* seedlings to 5 minutes salt treatment, PA originating from the PLC/DGK pathway was prevalent at concentrations of 50-500 mM NaCl, whereas only at very high concentrations (> 1000 mM), PLD was activated, giving rise to an additional PA increase (Chapter 6). These results were not only based on differential labelling experiments and transphosphatidylation, but also on the use of knockout (KO) mutants. Seedlings of *pldd* (T-DNA insertion mutant) showed total abrogation of the additional rise in PA at high concentrations. However, a seedling growth assay on saline media suggested decreased tolerance in the *pldac1* mutant, but not in *pldd* plants, suggesting that there is no direct relationship between the rapid PA response after brief exposure to stress and long-term responses in terms of acclimation and stress tolerance mechanisms. Currently, four different *Arabidopsis* PLDs have been suggested to play a role in growth under hypersalinic conditions [7, 8], but only one was found to be active in the immediate salt-induced PA response in seedlings.

Using T-DNA lines with insertions in or close to the DGK- and PLC-genes, an attempt was made to identify the involved isozymes. However, in none of the lines the salt-induced PA response was affected, suggesting that either an isozyme was involved for which no effective KO line was available, or that the suppression of one gene has lead to the upregulation of another, compensating for the loss. It will therefore be important to obtain KO mutants for every DGK and PLC gene and to check gene transcription levels of the other family members in these lines. To circumvent redundancy, multiple KOs might have to be generated for the total suppression of the PA response.

An interesting aspect of the phospholipid responses to hypersalinity is the finding that in *Arabidopsis* and in *Chlamydomonas*, different pathways are being activated at different levels of stress. This is obvious in the *Arabidopsis* PA response, but the other observed changes, viz. increases in PIP$_2$ and DGPP, also occurred at lower NaCl concentrations. Specific combinations of these putative LSMs could be associated with the activation of...
different protein targets such as protein kinases and small G-proteins, as was suggested for the *Chlamydomonas* salt response [9].

The finding that *Chlamydomonas* accumulated lysophosphatidic acid (LPA) under salt stress conditions, prompted us to investigate the origin of this lipid, which normally does not accumulate, but plays a role as intermediate in lipid biosynthesis (Chapter 5). Previous results, using pharmacological inhibitors, suggested that LPA increased due to the activation of a PA-hydrolyzing PLA$_2$ activity [10]. We addressed the question of LPA’s metabolic origin using a combination of differential labelling and fatty acid analysis (Chapter 5). The results lead us to propose a tentative model of different pools of PA, LPA, and their precursors, connected by pathways functioning in lipid biosynthesis or signalling. The accumulation of $\alpha$-linolenic acid-rich (18:3) LPA species argued for its formation via PLA$_2$-hydrolysis of a PA subpool with similar enrichment. This subpool is suggested to be formed by DGK through phosphorylation of PLC-generated DAG, reflecting the composition of $\alpha$-linolenic acid-rich PPI species. Interestingly, the suggested metabolic relationships argue for the co-localization of the enzymes involved, i.e. PLC, DGK and PLA$_2$. Moreover, the finding that the PLA$_2$ inhibitor aristolochic acid suppressed the LPA response and simultaneously induced an equivalent rise in DGPP, suggest that PA kinase competes for the same substrate and also has to be in the vicinity. The integrated analysis of such metabolic relationships and the subcellular localization of the phospholipids and enzymes by microscopic techniques will be one of the most challenging avenues of future phospholipid research.

Chapter 7 investigates the PA response of *Arabidopsis* seedlings and leaf discs to acute cold stress. Strikingly, also under this stress condition, PA increased within 2-10 min, with DGK activity being its most likely contributor. Moreover, the production of $^{32}$P-PA coincided with an equivalent decrease in $^{32}$P-PIP, suggestive of a PIP-hydrolyzing PLC activity that provides DAG. Notably, while PLD did not play a role in the early PA response, several isozymes, including PLD$\alpha$ [11] and PLD$\delta$ [12] have been implicated in long-term responses to cold with a role in cold acclimation. Moreover, the activity of PLD$\alpha$1 is stimulated at freezing temperatures, due to the loss of membrane integrity. The leading role for DGK in the early cold response is reflected in the finding that, of all genes encoding phospholipid metabolic enzymes found to be induced under cold stress, only *DGK1* gene was induced within the first 2 h of cold exposure [13]. Our attempt at identifying the involved PLC and DGK isozymes using T-DNA insertion lines was again confounded, indicating the lack of effective KO mutants and/or redundancy.

Over the last few years, a number of PA-binding proteins have been identified which are thought to be recruited to the membrane when and where PA is formed [14]. Their enzymatic activity can thus be regulated in a signal-dependent manner. With respect to hyperosmotic responses, two interesting candidate PA targets were protein kinases that are known to be activated by osmotic stress. They belong to the so-called SNF-related protein kinase 2 (SnRK2) family [15]. Current research is aimed at determining the protein
domains that are responsible for PA binding in these proteins but also other PA targets. By fusing such PA-binding domains to a fluorescent protein, PA biosensors are being generated. This will enable visualisation of PA pools \textit{in vivo}, using confocal microscopy, adding spatial information to our knowledge of metabolic pathways.

As the understanding of the organisation of PA metabolism increases, combining these data in mathematical models is becoming an interesting possibility. The metabolic network underlying the salt-induced accumulation of PA and LPA in \textit{Chlamydomonas}, described in Chapter 5, may be particularly suitable for making a biochemical systems model, comparable to the modelling of sphingolipid metabolism in \textit{Saccharomyces cerevisiae} [16]. It may generate testable hypotheses and thus be validated, and give insight into the regulation of PA metabolism under conditions of environmental stress.
References


