Activation of phospholipase D by calmodulin antagonists and mastoparan in carnation petals

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Abstract

An in vivo assay for phospholipase D (PLD; EC 3.1.4.4) activity, based on its transphosphatidylidation property, is described in detail and was used to study putative post-translational regulation mechanisms of PLD activity in carnation (Dianthus caryophyllus L.) petals. A variety of agents was applied to petal discs. The calmodulin (CaM) antagonists propranolol, N-(6-amino-hexyl)-5-chloro-1-naphthalenesulphonamide (W7) and N-(6-aminohexyl)-1-naphthalenesulphonamide (W5), stimulated PLD activity in a dose-dependent manner. EGTA partially inhibited the stimulation by the CaM antagonists. Erythrosin B, an inhibitor of CaM-dependent P-type Ca2+-ATPases, slightly stimulated PLD activity. The results suggest that part of the stimulation of PLD activity by CaM antagonists is due to an increased intracellular Ca2+-concentration. PLD activity was stimulated by mastoparan in a dose- and time-dependent manner. The signal-like activation kinetics suggests that mastoparan activates PLD (in)directly via a G protein.

Key words: Phospholipase D, CaM antagonists, mastoparan, Dianthus, calcium.

Introduction

Phospholipase D hydrolyses the phosphate ester between the phosphatidate moiety and the head group of glycerophospholipids. The enzyme was first discovered in carrot roots and spinach leaves (Hanahan and Chaikov, 1947) and has since been shown to be widely distributed throughout the plant kingdom (Quarles and Dawson, 1969; Heller, 1978). Despite its high activity in plants, the physiological function of PLD in growth and development is still not clear. PLD could be involved in phospholipid turnover that maintains cell viability and homeostasis (reviewed by Dawidowicz, 1987). Additionally, the observation that the phospholipid content of petals, fruits and leaves decreased during senescence (McArthur et al., 1964; Ferguson and Simon, 1973; Beutelmann and Kende, 1977; Thompson et al., 1982) led to the hypothesis that PLD initiated this lipid breakdown (Brown et al., 1990; Paliyath and Droillard, 1992). The idea was partly based on changes in PLD activity that were measured using in vitro assays (Herman and Chrispeels, 1980; Borochov et al., 1982; Salama and Pearce, 1993), but recently, it was shown that regulation of PLD activity is more complicated. During castor bean germination and leaf senescence, not only the total PLD content was changed, but also three isoforms were differentially expressed (Dyer et al., 1994; Ryu and Wang, 1995). Furthermore, the intracellular distribution of PLD changed, with increasing activity becoming associated with its substrate in membranes (Xu et al., 1996). This suggests that post-translational mechanisms contribute to the regulation of PLD activity.

Post-translational regulation mechanisms apply to PLD involved in signalling. A recent study of the green alga Chlamydomonas illustrated that G-protein activation triggered immediate increases in PLD activity (Munnik et al., 1995), generating PtdOH which is a second messenger in animal cells (Liscovitch and Cantley, 1994, 1995), and which had dramatic effects on Chlamydomonas, causing deflagellation (Munnik et al., 1995).

In order to investigate the different functions of PLD in higher plants, activity must be measured in vivo, and in a preliminary study it was shown that this is possible with live carnation petals by using the transphosphatidyl-
infiltration. After release of the vacuum, incubations were
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labelling buffer containing 3.5 \( \mu \text{Ci} \) [\(^{3}\text{H}\)] was added to the labelled buffer. Separate petal discs were labelled at room temperature in 250 \( \mu \text{l} \) of a labelling buffer containing 3.5 \( \mu \text{Ci} \) [\(^{3}\text{H}\)] palmitic acid by vacuum-infiltration. After release of the vacuum, incubations were started by the simultaneous or successive addition of \( \alpha \)-butanol (1 vol% in 250 \( \mu \text{l} \) of the labelling buffer) and the test reagents (stock solutions of DL-propranolol, W5, W7, mastoparan, and Masl7 in deionized water) as specified in the figure legends. Incubations were continued for the times indicated, the reactions quenched and the lipids extracted as described above.

A mixture of unlabelled PtdBut, PtdOH, and PtdCho in CHCl\(_{3}\)-MeOH (9:1, v/v) was added to the dried samples. The total samples were chromatographed using three solvents consecutively, with intermediate drying. First, CHCl\(_{3}\) was used to reduce \(^{3}\text{H}\)-counts in the background due to the presence of unincorporated palmitic acid in the samples. Second, acetone was used to remove monogalactosyl diglyceride which otherwise co-migrated with PtdBut (\( R_{p} \) value PtdBut, 0.43). The TLC plate was developed using the ethyl acetate-isooctane solvent described above to separate PtdBut and PtdOH from the rest of the phospholipids.

PLD activity was presented as the formation of PtdBut which is expressed as the level of \(^{3}\text{H}\)-counts in PtdBut as a percentage of the label recovered from the structural phospholipids (SPL), PtdCho, PtdIns, PtdEtn, and PtdGro. The \(^{3}\text{H}\)-counts in the PtdBut fractions were corrected for those migrating at the position of PtdBut in control samples incubated without \( \alpha \)-butanol. Lipid fractions isolated from petal discs incubated for 1 h with \([\text{H}]\text{palmitate in the presence or absence of } \alpha \text{-butanol (0.5%) contained on average (n=6\pm12, SE) : SPL, 216 \pm 24,000 dpm; PtdBut (not corrected), 1,180 \pm 120 dpm; background PtdBut, 470 \pm 45 dpm.}

**Materials and methods**

**Plant material**

Carnation (Dianthus caryophyllus L.) flowers cv. White Sim were obtained from a commercial grower and transported dry to the laboratory. Stems were re-cut to 20 cm and placed in deionized water under controlled environmental conditions (Woltering et al., 1993a). Just before use, discs (0.8 cm diameter, approximately 15 mg fresh weight) were cut from the middle part of the petals. Only the petals of the outer whorl were used.

**Materials**

[9,10 \( (\text{n})^{3}\text{H}\)] Palmitic acid (40–60 Ci mmol\(^{-1}\)) was obtained from Amersham (Buckinghamshire, UK). The analytical grade organic solvents and TLC silica 60 on plastic sheets were from Merck (Darmstadt, Germany). Phospholipid standards, phospholipase D (cabbage), erythrosin B, DL-propranolol, W5, W7, and mastoparan (Vespula lewisii) were purchased from Sigma (St Louis, MO, USA). Synthetic mastoparan and mastoparan \( \alpha \)-butanol (Vespula lewisii) were purchased from Bachem (Bubendorf, Switzerland). A standard of PtdBut was prepared by conversion of egg-yolk PtdCho into PtdBut by phospholipase D in the presence of 5\% (v/v) \( \alpha \)-butanol (Comfurius and Zwaal, 1977).

**Lipid labelling, extraction and analyses**

Petal discs (a maximum of 10) were labelled with \([\text{H}]\) palmitic acid (10 \( \mu \text{Ci} \)) in 2 ml labelling buffer (25 mM MES, 0.05% Tween 20, pH 5.5) by vacuum-infiltration (Munnik et al., 1994). After 5 min, the vacuum was released and the discs were incubated at room temperature for the times indicated. Incubations were stopped by immersing each petal disc in 600 \( \mu \text{l} \) of CHCl\(_{3}\)-MeOH: HCl (100:100:1, by vol.) and freezing the samples in liquid nitrogen. Lipids were directly extracted after thawing and the petal discs were re-extracted with 450 \( \mu \text{l} \) of the same solution. Lipid fractions were pooled and a two-phase separation induced by the addition of 500 \( \mu \text{l} \) CHCl\(_{3}\) and 375 \( \mu \text{l} \) 0.9\% (w/v) NaCl, 1 mM EGTA. The organic lower phase was washed once with 375 \( \mu \text{l} \) MeOH: H\(_{2}\)O: HCl (50:50:1, by vol.). Lipid extracts were dried under a stream of nitrogen at 30 °C and stored at −20 °C until further use.

For TLC analyses, lipids were dissolved in CHCl\(_{3}\)-MeOH (9:1, v/v) and samples applied to silica TLC plates which were then developed in the organic upper phase of ethyl acetate:iso-octane: HAE: H\(_{2}\)O (13:2:3:10, by vol.). Lipid spots were revealed with iodine vapour and identified by co-migration with unlabelled lipid standards. The \( R_{p} \) values for the lipids were: PtdCho and PtdIns, 0.01; PtdEtn and PtdGro, 0.06; PtdOH, 0.24; monogalactosyl diacylglycerol, 0.44. Spots of interest were excised and their radioactivity was determined by liquid scintillation counting.

**In vivo Phospholipase D assay**

Separate petal discs were labelled at room temperature in 250 \( \mu \text{l} \) labelling buffer containing 3.5 \( \mu \text{Ci} \) [\(^{3}\text{H}\)] palmitic acid by vacuum-infiltration. After release of the vacuum, incubations were started by the simultaneous or successive addition of \( \alpha \)-butanol (1 vol% in 250 \( \mu \text{l} \) of the labelling buffer) and the test reagents (stock solutions of DL-propranolol, W5, W7, mastoparan, and Masl7 in deionized water) as specified in the figure legends. Incubations were continued for the times indicated, the reactions quenched and the lipids extracted as described above.

Measurement of ion leakage

Excised petals from the outer whorl were placed with their cut base in 3 ml of a 4% \( \alpha \)-butanol solution for 24 h. Thereafter, ion leakage was measured as described by Woltering et al. (1993b). Briefly, 10 petals were immersed in 30 ml deionized water and gently shaken. Conductivity of the diffusate was measured between 1 and 3 h. The total conductivity was determined after two cycles of freezing and thawing. Conductivity was expressed as the percentage ion leakage per hour relative to the total conductivity of the petals.

**Results**

\([\text{H}]\) Palmitic acid labelling of lipids

Carnation petal discs were incubated with \([\text{H}]\) palmitic acid, their lipids extracted at regular intervals, and the lipids separated by TLC. After 24 h, about half of the \(^{3}\text{H}\)-label in the lipid extract was present as phospholipids.

Most was incorporated into PtdCho plus PtdIns (56%) and PtdEtn plus PtdGro (42%). Approximately 1.3% of the label was recovered as PtdOH. The rest of the \(^{3}\text{H}\)-label was recovered as glycolipids (3%) or migrated nearer the solvent front, probably representing diacylglycerols, triacylglycerols and unincorporated palmitic acid.

Incorporation of \(^{3}\text{H}\) into phospholipids increased linearly during the first 8 h, usually reached a maximum within 24 h (Fig. 1A) and then remained at the same level up to 48 h, suggesting that isotopic equilibrium had been reached. The \(^{3}\text{H}\)-label was incorporated into PtdOH and the structural phospholipids in the same relative proportion during the whole labelling period (Fig. 1B), indicat-
In vivo PLD activity

Fig. 1. Incorporation of \([^{3}\mathrm{H}]\)palmitate into the phospholipids of carnation petals. Petal discs were labelled with \([^{3}\mathrm{H}]\)palmitate (5 \(\mu\)Ci ml\(^{-1}\)) for increasing times up to 24 h. Lipids were extracted, separated by TLC and analysed as described in Materials and methods. (A) Data are expressed as the sum of the radioactivity in PtdCho, PtdIns, PtdEtn, and PtdGro. The experiment was repeated once with essentially similar results. (B) Data are expressed as the relative \(^{3}\mathrm{H}\) incorporation per phospholipid species, (O) PtdOH; (O) PtdCho plus PtdIns; (O) PtdEtn plus PtdGro. The results are means±SE of 2-17 values from different experiments. PL, phospholipids.

Fig. 2. Formation of PtdBut in control (O) and propranolol-treated (O) petal discs as a function of the incubation time (A) and concentration (B) of n-butanol. Petal discs were incubated with \([^{3}\mathrm{H}]\)palmitate (3.5 \(\mu\)Ci/250 \(\mu\)l) and propranolol (5 mM) for 2 h (A) and 1 h (B). (A) n-Butanol (0.5\%) was present during the last period of the incubation for the indicated times. (B) n-Butanol was present at the indicated concentrations for 1 h. The results are means±SE, \(n\geq 2\) from different experiments. SPL, structural phospholipids.

The local anaesthetic propranolol, a CaM antagonist, (Volpi et al., 1981), was tested for its effect on in vivo PLD activity. When 0.5\% n-butanol was added to petal discs pretreated with 5 mM propranolol, PtdBut formation was rapidly elevated above the control and increased for at least 60 min (Fig. 2A, open circles). The increase was linear with increasing n-butanol concentrations up to 2\% (Fig. 2B). Membrane permeability was not adversely affected in as much as treatment with 4\% n-butanol for 24 h did not increase ion leakage (conductivity: control petals, 0.42\% h\(^{-1}\), SE±0.02; n-butanol-treated petals, 0.48\% h\(^{-1}\), SE±0.07).

PtdBut formation was measured after 1 or 17 h of prelabelling with \([^{3}\mathrm{H}]\)palmitate (see Fig. 1A for relative incorporation levels) using 0.5\% n-butanol and 5 mM propranolol. After 1 h under both labelling conditions,
Fig. 3. Time-course of PLD-dependent PtdBut formation upon stimulation with propranolol. Petal discs were stimulated with propranolol (5 mM) for increasing times up to 2 h. Labelling with \(^{3}H\)palmitate was carried out in the presence of \(\pi\)-butanol (0.5%) for 2 h. The results are means±SE of 3–10 values from different experiments. SPL, structural phospholipids.

approximately 2.4% of the phospholipid label was converted into PtdBut. Therefore, in subsequent experiments, PLD activity was routinely measured using petal discs incubated with 0.5% \(\pi\)-butanol and \(^{3}H\)palmitate during 1 h.

Addition of 5 mM propranolol to petal discs rapidly stimulated the formation of \(^{3}H\)PtdBut and after 2 h of treatment \(^{3}H\)PtdBut accumulated up to 10-fold (Fig. 3). The effect was dose-dependent and increased up to 10 mM (Fig. 4A). Higher concentrations of propranolol inhibited the incorporation of \(^{3}H\)palmitate into the structural phospholipids (data not shown).

The naphthalenesulphonamides W7 and W5, two other CaM antagonists, also stimulated PLD activity in a dose-dependent manner (Fig. 4B). W7 (closed circles) was found to be more active than W5 (open circles), which correlated well with their order of potency (Hidaka and Tanaka, 1983). Higher concentrations of these CaM antagonists again inhibited the incorporation of label into the structural phospholipids.

**Mastoparan stimulates PLD activity**

The G protein activator mastoparan was used to get insight into the possible involvement of G proteins in the regulation of PLD activity in carnation petals. Mastoparan stimulated PLD activity in a concentration- and time-dependent manner (Fig. 5A, B). At 25 \(\mu\)M, activity was stimulated more than 5-fold. Mas17, a mastoparan analogue which is a far less potent activator of G proteins (Higashijima et al., 1990), did not significantly stimulate PLD activity (Fig. 5A). The effect of mastoparan was very rapid, half-maximal activity being reached within 5 min, producing kinetics of stimulation that were very different to those found for propranolol (Fig. 3).

Besides an increase in \(^{3}H\)PtdBut formation, treatment of petal discs with either mastoparan or propranolol also resulted in a stimulation of the percentage of \(^{3}H\)-label in PtdOH. Interestingly though, mastoparan and propranolol differed in the ratio of PtdBut/PtdOH formation. To understand this point one must appreciate that the primary alcohol competes with water to accept the phosphatidyl group. Thus the PtdBut to PtdOH ratio is determined by the affinity of PLD for the acceptor and its local concentration. Even when stimulated, the PtdBut to PtdOH ratio is expected to be constant. Indeed, that was found for both stimulation by mastoparan and propranolol (Fig. 6). However, the PtdBut : PtdOH ratio for mastoparan was 1:5.5 (SE±0.1, \(n=2\), data were derived from two series of concentrations of mastoparan), while that for propranolol was 1:3.2 (SE±0.3, \(n=8\), data were derived from a series of concentrations of propranolol and kinetic experiments).
Discussion

It has been shown that phosphatidylolation of primary alcohols can be used as a measure of in vivo PLD in plant tissues. The advantages of this assay are first that phosphatidylalcohols are stable products while PtdOH and, for example, choline from PtdCho, are not. Second, PLD activity can be determined in vivo because the formation of phosphatidylalcohols is specific for PLD activity while that of PtdOH is not. That transphosphatidylation is indeed a property of PLD was demonstrated by introducing a PLD gene from *Ricinus communis* into *E. coli* and showing that only when the gene was expressed were extracts of the bacterium able to hydrolyse phosphatidylcholine and transphosphatidylate ethanol (Wang et al., 1994). Similar results have recently been obtained using a human PLD gene expressed in insect cells (Hammond et al., 1995).

In order to use the transphosphatidylolation assay for PLD activity, the phosphatidyl moiety of the PLD substrate must be labelled. If the substrate has not been determined, this means that all structural phospholipids must be effectively labelled. Incubating discs in [3H]palmitate achieves this goal, and in our system it was incorporated into all major phospholipids at equal rates. When 0.5% n-butanol was then added, the formation of PtdBut increased linearly with time. While it is tempting to use higher concentrations of n-butanol to facilitate the detection of PtdBut, one should realize that alcohols can themselves activate signalling enzymes such as PLC and PLD (Hoek et al., 1992; Musgrave et al., 1992; Munnik et al., 1995). Higher concentrations of n-butanol added to the carnation petals certainly increased PtdBut formation, but whether this reflects PLD activation or just an increase in substrate concentration was not tested.

The ability to measure PLD activity in vivo allowed the study of some regulatory aspects of PLD. The CaM antagonists W7, W5, and propranolol stimulated PLD activity to a varying extent. PLD itself is not a CaM-regulated enzyme, but CaM-dependent enzymes in plants have been described. CaM antagonists have been shown to raise cytosolic calcium levels in plant protoplasts (Gilroy et al., 1987), perhaps by inhibiting CaM-dependent P-type Ca^{2+}-ATPases in the plasma- and ER membranes (Dieter and Marme, 1980, 1981; Briskin, 1990; Evans et al., 1991; Askerlund and Evans, 1992). Since in vitro PLD activity has been shown to be calcium-dependent (Heller, 1978) and since the recently cloned PLD genes from plants contain a potential calcium-
binding domain (Wang et al., 1994; Ueki et al., 1995), calcium could be a general trigger that activates PLD. Propranolol, W7 and W5 were effective at relatively high concentrations ranging from 0.5 to 5 mM, but their order of potency (W7 > propranolol ≈ W5) correlated with their IC₅₀ for CaM-dependent phosphodiesterase activity (Volpi et al., 1981; Hidaka and Tanaka, 1983), suggesting that all three worked via CaM. The fact that EGTA (5 mM) partially inhibited propranolol-stimulated PLD activity (to 70% of the control) suggests that part of the effect of propranolol is due to an influx of extracellular Ca²⁺. Additionally, the significant stimulation of PLD by erythrosin B (50 µM, 1.6-fold increase), a specific inhibitor of P-type Ca²⁺-ATPases (Rasi-Caldogno et al., 1989; Askerlund and Evans, 1992), implicates calcium as the common denominator in all these responses. This is in agreement with a recent study where it is proposed that activation of PLD upon wounding of castor bean leaves is mediated by an increase in cytoplasmic Ca²⁺ (Ryu and Wang, 1996). Intracellular Ca²⁺-measurements will be needed to provide conclusive evidence about a possible role of Ca²⁺ in regulation of PLD in vivo. Besides specific effects of the CaM antagonists, aspecific effects such as perturbation of the membranes by these lipophilic molecules might contribute to a stimulation of PLD activity.

Mastoparan also stimulated PLD activity in carnation petals. It is thought to mimic the peptide domain of agonist-bound receptors that activates G proteins (reviewed by Ross and Higashijima, 1994). As such it could increase the intracellular Ca²⁺-concentration via a G protein activated Ca²⁺-channel or via PLC. There are several reports that mastoparan stimulates PLC activity in plants (Quarmby et al., 1992; Legendre et al., 1993; Drøbak and Watkins, 1994; Quarmby and Hartzell, 1994; Cho et al., 1995). By increasing the production of inositol-1,4,5-triphosphate, calcium can be released from intracellular stores and this could again stimulate PLD activity. Another possibility is that PLD is directly coupled to a G protein. This could account for the signal-like activation kinetics and is in agreement with the recent study of PLD activation in the alga Chlamydomonas (Munnik et al., 1995), where it was shown that treatments that are known to raise the calcium concentration and cause deflagellation, did not activate PLD, whereas mastoparan and other G protein activators did activate PLD. Lastly, mastoparan could directly activate PLD as was indicated recently for animal cells (Mizuno et al., 1995).

Both the kinetics of stimulation and the ratio of PtdBut:PtdOH formation for mastoparan and propranolol were different. The differences in kinetics could reflect different mechanisms of PLD stimulation or could be determined by different uptake rates or physical properties such as membrane affinity. The different ratios of PtdBut to PtdOH indicate that mastoparan and propranolol could stimulate or inhibit enzymes that affect PtdOH levels such as PLC in combination with diacylglycerol kinase, PtdOH kinase, and PtdOH phosphatase (Munnik et al., 1996). An alternative explanation is that mastoparan and propranolol activate two different isoforms. The idea of different PLD isoforms in higher plants has already been established in castor bean based on immunoblotting and activity assays (Dyer et al., 1994). In addition, three cDNAs have recently been identified in Arabidopsis (Dyer et al., 1995). The possibility that PLD isoforms can be differentially activated by CaM antagonists and mastoparan is new, exciting and deserves more attention.

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