Plant Physiol. (1995) 107: 953–962

Plant Microsomal Phospholipid Acyl Hydrolases Have Selectivities for Uncommon Fatty Acids[1]

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Developing endosperms and embryos accumulating triacylglycerols rich in caproyl (decanoyl) groups (i.e. developing embryos of *Cuphea procumbens* and *Ulmus glabra*) had microsomal acyl hydrolases with high selectivities toward phosphatidylcholine with this acyl group. Similarly, membranes from *Euphorbia lagascae* and *Ricinus communis* endosperms, which accumulate triacylglycerols with vernoleate (12-epoxy-octadeca-9-enoate) and ricinoleate (12-hydroxy-octadeca-9-enoate), respectively, had acyl hydrolases that selectively removed their respective oxygenated acyl group from the phospholipids. The activities toward phospholipid substrates with epoxy, hydroxy, and medium-chain acyl groups varied greatly between microsomal preparations from different plant species. Epoxidated and hydroxylated acyl groups in *sn*-1 and *sn*-2 positions of phosphatidylcholine and in *sn*-1-lysophosphatidylcholine were hydrolyzed to a similar extent, whereas the hydrolysis of caproyl groups was highly dependent on the positional localization.

A number of different phospholipases, both intracellular and secreted, have been characterized in microorganisms and animal tissues, and the literature in the area is extensive (reviewed by Van den Bosch, 1980, 1982; Waite, 1987). However, very little is known about phospholipid turnover in plants. A ubiquitously occurring phospholipase D with unknown physiological function has been known for a long time (Hanahan and Chaikoff, 1947; Wang et al., 1993), and more recent studies have demonstrated the presence of a phospholipase C specific for phosphorylated phosphatidyl-inositols (Sandelius and Sommarin, 1990). Only a few studies of phospholipases of A and B types have been reported in plants (Kim et al., 1994). However, several acyl hydrolases with activity not only toward phospholipids but also toward glycolipids, sulfolipids, monoolein, and diolein have been studied in several plant tissues (Huang, 1987).

A phospholipase specific for ricinoleoyl (12-hydroxy-octadeca-9-enoyl) groups was reported in microsomes from developing endosperms of *Ricinus communis* (Bafor et al., 1991). The enzyme seems to play a role in the liberation of newly synthesized ricinoleate from the phospholipids, thereby supplying triacylglycerol synthesis with acyl substrate and keeping the concentration of ricinoleate in the membranes low. High amounts of hydroxy fatty acids in the membranes would probably severely disturb the structure and functions of the membranes. Microsomes from plants that do not accumulate ricinoleate in the seed storage fat were also found to have acyl hydrolase activity with specificity for ricinoleate. From these results it was proposed that this phospholipase is involved in a general membrane repair mechanism (Bafor et al., 1991), as postulated for mammalian phospholipase A_2 (Van Kuijk et al., 1987). This idea was further supported by a later study that showed that hydroperoxide-linoleate was also hydrolyzed from PC by plant microsomes (Banas et al., 1992). *Euphorbia lagascae* endosperm accumulates vernolic acid (12-epoxy-octadeca-9-enoic) in high amounts in the triacylglycerols, but the concentration in the seed membranes is not more than a few percent (Bafor et al., 1993). It was found that microsomal membranes prepared from developing seed of *E. lagascae* catalyzed a P450-dependent epoxidation of linoleoyl-PC and a subsequent hydrolysis of the newly synthesized oxygenated acyl groups from the phospholipids (Bafor et al., 1993).

To more fully characterize the acyl and the stereo specificities of these microsomal phospholipid acyl hydrolases, we have compared the activities, specificities, and selectivities of microsomal enzymes from developing endosperms of *E. lagascae* and *R. communis*, which accumulate two different oxygenated acyl groups, with those from developing embryos from *Cuphea procumbens* and *Ulmus glabra*, which accumulate medium-chain acyl groups, and with those from developing embryos from low erucic rape (*Brassica napus*), which accumulate only acyl groups common for both membranes and triacylglycerols.

[1] This research was supported by The Swedish Council for Forestry and Agricultural Research, the Swedish Natural Science Research Council, the Swedish Farmer's Foundation for Agricultural Research, the Skånska Lantmännen Foundation, and Stiftelsen Svensk Oljeväxtforskning.

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Abbreviations: BCA, bicinchoninic acid; CHAPS, 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

MATERIALS AND METHODS

Materials

CHAPS was purchased from Boehringer Mannheim (Mannheim, Germany) and dimethylacetal (Methyl 8) was from Pierce (Rockford, IL). The following chemicals were obtained from Sigma: BSA (fatty acid free), ATP, CoASH, NADH, NADPH, capric (decanoic, 10:0) acid, lauric (dodecanoic, 12:0) acid, palmitic (hexadecanoic, 16:0) acid, oleic (octadeca-9-enoic, 18:1) acid, linoleic (octadeca-9,12-dienoic, 18:2) acid, ricinoleic (12-hydroxy-octadeca-9-enoic) acid, dipalmitoyl-PC (di 16:0-PC), palmitoyl-LPC (*sn*-1-palmitoyl-glycero-phosphocholine, 16:0-LPC), LPE (*sn*-1-acyl-glycerol-phosphoethanolamine, prepared from egg yolk), phospholipase A_2 (from *Naja naja* snake venom), triacylglycerol lipase (from *Rhizopus arrhizus*), phospholipase D (from *Streptomyces chromofuscus*) and [1-^{14}C]10:0 acid. Amersham (Buckinghamshire, UK) provided the following [1-^{14}C]fatty acids: 12:0, 16:0, 18:0, 18:1, 18:2.

Vernolic (*cis*-12-epoxy-octadeca-9-enoic) acid was prepared from methyl esters of *Euphorbia lagascae* seed triacylglycerols as follows: vernoleoyl methyl esters were separated from other fatty acid methyl esters by TLC on silica gel plates developed in hexane:diethyl ether:acetic acid (85:15:1, v/v/v) and eluted from the gel in methanol:chloroform (2:1, v/v). The vernoleoyl methyl esters were hydrolyzed by treatment with 0.4 M KOH in water:methanol (4:1, v/v) at 80°C for 120 min. After subsequent neutralization with acetic acid, the free vernolic acid was extracted into $CHCl_3$ and purified by TLC on silica gel plates developed in hexane:diethyl ether:acetic acid (70:30:1, v/v/v) and eluted from the gel with methanol:chloroform (2:1, v/v). [1-^{14}C]Ricinoleic acid was obtained by biochemical conversion of [1-^{14}C]oleate by the microsomal Δ^{12}hydroxylase from *Ricinus communis* endosperm as described by Bafor et al. (1991). [1-^{14}C]Vernoleate was synthesized by epoxidation of [1-^{14}C]18:2 by the microsomal Δ^{12}epoxidase from *E. lagascae* endosperm as follows: microsomal preparations from developing *E. lagascae* endosperm (10 mg of microsomal protein), prepared according to Bafor et al. (1993), were incubated with 2 μmol of [^{14}C]18:2, 80 mg of BSA, 25 μmol of ATP, 8 μmol of CoASH, 25 μmol of $MgCl_2$, and 20 μmol of NADPH in 0.1 M potassium phosphate buffer, pH 7.2, in a total volume of 4 mL for 12 h at 25°C. After chloroform extraction of the incubation mixture, the lipids in the chloroform extract were methylated by treatment with dimethylacetal and sodium methoxide (Bafor et al., 1993). Free [^{14}C]vernolic acid was prepared from the methyl ester mixture using the same procedure as described above for unlabeled vernolic acid. The yield of [^{14}C]vernolic acid was 10 to 15% of added [^{14}C]18:2 substrate.

Synthesis of [^{14}C]Acyl-Phospholipid Substrates

Before being used in [^{14}C]acyl-PC synthesis, all radioactive fatty acids were adjusted to a specific radioactivity of about 2000 dpm/nmol by addition of unlabeled fatty acids. The TFA anhydride method described by Kanda and Wells (1981) was used for the synthesis of the saturated and oleoyl *sn*-2-[^{14}C]acyl-PC substrates, from radioactive fatty acids and commercial *sn*-1–16:0-LPC. Corresponding *sn*-1-[^{14}C]acyl-PC was prepared with the same method using *sn*-2–16:0-LPC. The *sn*-2–16:0-LPC was prepared by treatment of di-16:0-PC with triacylglycerol lipase for 30 min, and rapid separation and isolation of the *sn*-2-acyl-LPC by TLC on silica gel plates developed in chloroform:methanol: acetic acid:water (170:30:20:7, v/v/v).

The TFA anhydride method could not be used for PC synthesis with fatty acids containing hydroxy or epoxy groups. Therefore, *sn*-2-[^{14}C]vernoleoyl-PC and *sn*-2-[^{14}C]ricinoleoyl-PC were prepared by enzymatic acylation of *sn*-1–16:0-LPC using the microsomal acyl-CoA:LPC acyltransferase from developing sunflower seeds according to Banas et al. (1992) but with the following alterations of the reaction mixture. Radioactive free acid (800 nmol), 24 mg of BSA, 40 μmol of ATP, 12 μmol of CoASH, 40 μmol of $MgCl_2$, 1.2 μmol of LPC, and sunflower microsomal membranes (equivalent to 0.3 mg of microsomal protein) were incubated in 0.1 M potassium phosphate buffer, pH 7.2, in a final volume of 2 mL for 2 h at 30°C.

sn-1-[^{14}C]ricinoleoyl- and *sn*-1-[^{14}C]vernoleoyl-PC were prepared by first treating the *sn*-2-[^{14}C]ricinoleoyl- and *sn*-2-[^{14}C]vernoleoyl-PC with triacylglycerol lipase and isolating the resulting *sn*-2-[^{14}C]acyl-LPC. The *sn*-2-LPC was then allowed to isomerize to the more stable *sn*-1-LPC by leaving it in methanol solution (saturated with N_2) for 1 week at room temperature. The *sn*-1-[^{14}C]ricinoleoyl-PC and *sn*-1-[^{14}C]vernoleoyl-PC were then synthesized by enzymatic acylation of corresponding *sn*-1-[^{14}C]acyl-LPC with oleate from oleoyl-CoA using the microsomal acyl-CoA:acyltransferase from sunflower (Banas et al., 1992).

All [^{14}C]acyl-PC substrates were checked for the stereospecific distribution of their [^{14}C]acyl groups by treatment with phospholipase A_2 (from *N. naja*) and subsequent determination of the distribution of radioactivity between the free fatty acid and the LPC (Griffiths et al., 1985). The percentage of radioactivity located in the *sn*-2 position of the *sn*-2-[^{14}C]acyl-PC substrates ranged from 89 to 98.5%. The percentage of label in the *sn*-1 position of the *sn*-1-[^{14}C]acyl-PC substrates varied more. The following percentages of radioactivity were associated with the *sn*-1 position for the various substrates: *sn*-1-[^{14}C]10:0-PC, 87%; *sn*-1-[^{14}C]16:0-PC, 61%; *sn*-1-[^{14}C]18:1-PC, 74%; *sn*-1-[^{14}C]vernoleoyl-PC, 78%; *sn*-1-[^{14}C]ricinoleoyl-PC, 53%.

sn-1-[^{14}C]acyl-LPC substrates were prepared by treating the corresponding *sn*-1-[^{14}C]acyl-PC with the highly positional specific phospholipase A_2 from *N. naja* and the resulting *sn*-1-LPC was then isolated. It should thus be noted that the [^{14}C]LPC formed were nearly exclusively *sn*-1, although the [^{14}C]PC from which they were prepared had varying proportions of [^{14}C]acyl groups also in position *sn*-2. *sn*-2-[^{14}C]10:0-PE was synthesized by chemical acylation of LPE (prepared from egg yolk) by using the TFA anhydride method (Kanda and Wells, 1981). *sn*-2-[^{14}C]acyl-PA was prepared by treatment of *sn*-2-[^{14}C]acyl-PC with phospholipase D from *S. chromofuscus*.

Since microsomal membranes from developing sunflower seeds were used in the preparation of [^{14}C]ricino-

leoyl-PC and [^{14}C]vernoleoyl-PC, these substrates contained a certain amount of sunflower PC. To standardize the [^{14}C]PC substrates, PC isolated from sunflower seed microsomes were added to the different synthesized [^{14}C]PC to yield a similar mixture of 55% [^{14}C]acyl-PC and 45% of sunflower microsomal PC for all substrates. These mixtures were then used in the phospholipid acyl hydrolase assays.

Plant Material and Microsomal Preparations

Cuphea procumbens, *R. communis*, and *E. lagascae* plants were grown from seeds in a photoperiod of 16 h light at 26°C and 8 h dark at 20°C. The seeds were harvested at the stage of rapid triacylglycerol accumulation, i.e. about 16, 35, and 25 DAF, respectively. Developing *Ulmus glabra* seeds were harvested from local trees at a stage when the embryos had not reached full size and when the seed coats were still green. The embryos or endosperms from all species were removed from their seed coats before further manipulations. Microspore-derived embryos of *Brassica napus* (var Topas) were cultivated according to Wiberg et al. (1991) and harvested after 15 d in shake cultures. Embryos at this stage had a very rapid triacylglycerol accumulation with a fatty acid profile virtually indistinguishable from the zygotic embryos (Wiberg et al., 1991).

Plant tissues were ground in mortar with 10 parts (v/w) of 0.1 M potassium phosphate buffer, pH 7.2, containing 0.33 M Suc. The homogenates were filtered through two layers of Miracloth, diluted 10-fold with fresh grinding medium, and centrifuged at 20,000 *g* for 10 min. The supernatants were filtered through Miracloth and centrifuged at 105,000 *g* for 90 min. The resulting microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.2, and stored at −80°C until used.

Assays

The standard assay of phospholipid acyl hydrolases contained 3.6 mM CHAPS, 4 nmol of [^{14}C]acyl-phospholipid (dispersed in CHAPS by sonication), microsomal membranes (equivalent to 25 nmol of microsomal PC or approximately 100 μg of microsomal protein), 5 mM EGTA in 0.1 M potassium phosphate buffer, pH 7.2, in a final volume of 200 μL. The assay was started by the addition of the microsomal membranes. Incubations were performed at 30°C with constant shaking for the times indicated in the tables and figures. Reaction mixtures, except those containing [^{14}C]acyl-LPC substrates, were terminated by adding 1 mL of 0.15 M acetic acid. The lipids were extracted into chloroform by the addition of 3.75 mL of methanol:chloroform (2:1, v/v) followed by 1.25 mL of chloroform and 0.8 mL of water. The method is a modified version of Bligh and Dyer (1959). Since LPC with medium-chain or oxygenated acyl groups were poorly extracted into chloroform, incubation mixtures with [^{14}C]acyl-LPC substrates were instead terminated by the addition of 2 mL of water-saturated *n*-butanol containing 1% acetic acid. After addition of 1 mL of water and phase separation, the butanol fractions were removed and the water fractions were reextracted with an additional 2 mL of the water-saturated *n*-butanol solution.

Analytical Procedures

The chloroform and butanol extracts were evaporated to dryness under N_2 and the residue was dissolved in a small volume of chloroform. LPC, PC, PA, and free fatty acid in the lipid extracts were separated on TLC plates (Merck & Co., Rahway, NJ; silica 60) developed in chloroform:methanol:acetic acid:water (170:30:20:7, v/v/v/v). Unsaturated lipids were located by staining with I_2 vapor, whereas saturated lipids were visualized by spraying with water. Lipid areas and the rest of the lanes were removed from the plates and assayed for radioactivity by scintillation counting with toluene:ethanol (2:1, v/v) solution and 0.4% 2(1-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole as scintillant.

Phospholipid and free fatty acid acyl quality and quantity were determined by GC of their methyl esters and with methyl-heptadecanoate as internal standard. Protein was measured with the BCA protein reagent (Pierce) after treatment of the microsomal preparations with 0.1% SDS and with BSA used as standard.

Reproducibility of the Results

All assays were performed in at least duplicate samples. Good reproducibilities were obtained between duplicate samples in the radioactive measurements of the different lipid classes (less than ±5% variation between determinations). The variation between replicate samples in the quantification of unlabeled acyl groups in PC and free fatty acids was considerably greater, and the statistical analysis of these data is presented in "Results."

RESULTS

Acyl Group Composition of the Different Tissues

The fatty acid composition of the triacylglycerols and of PC in the developing embryos or endosperms of the five plant species used are shown in Table I. *B. napus* embryos accumulate only fatty acids that are common for both membrane lipids and triacylglycerols (i.e. 16:0, 18:0, 18:1, 18:2, and 18:3). The endosperms or embryos of the other plant species accumulate triacylglycerol with fatty acids, which are nearly absent in the membrane lipids. *R. communis* and *E. lagascae* endosperm triacylglycerols have high amounts of oxygenated acyl groups, ricinoleate and vernoleate, respectively, whereas *U. glabra* and *C. procumbens* embryos have triacylglycerols rich in capric acid.

The Effect of CHAPS and Substrate Concentrations on *sn*-2-Acyl-PC Hydrolysis

Microsomal preparations of *R. communis* endosperms were incubated for 2 h in the presence or absence of CHAPS with either *sn*-2-[^{14}C]ricinoleoyl-PC or *sn*-2-[^{14}C]-oleoyl-PC at different concentrations (Fig. 1). [^{14}C]Oleoyl-PC was, at all concentrations and regardless of addition of CHAPS, utilized poorly by the acyl hydrolase(s). In the

Table I. *Fatty acid composition of triacylglycerols and PC isolated from developing embryos of B. napus, U. glabra, and C. procumbens and from developing endosperms of E. lagascae and R. communis*

The fatty acid distribution is expressed in mol %.

Fatty Acid	*Brassica napus*		*Ulmus glabra*		*Cuphea procumbens*		*Ricinus communis*		*Euphorbia lagascae*	
	TAG[a]	PC	TAG	PC	TAG	PC	TAG	PC	TAG	PC
Uncommon acyl groups										
Vernoleate	0	0	0	0	0	0	0	0	57	4
Ricinoleate	0	0	0	0	0	0	85	5	0	0
8:0	0	0	11	0	1	0	0	0	0	0
10:0	0	0	68	2	89	0	0	0	0	0
Common acyl groups										
16:0	4	9	4	14	2	19	2	18	4	12
18:1	63	54	5	20	3	30	5	31	22	9
18:2	18	25	4	53	1	45	6	37	10	61
18:3	13	12	0	6	0	1	1	3	0	0
Others	2	9	8	5	4	6	1	6	7	7

[a] TAG, Triacylglycerol.

absence of CHAPS, [^{14}C]ricinoleic acid was formed from [^{14}C]ricinoleoyl-PC to a small extent. Inclusion of 3.6 mM CHAPS brought about a 20-fold increase in the liberation of [^{14}C]ricinoleic acid. The hydrolysis was proportional to a PC concentration of 40 μM. Incubations with CHAPS of concentrations lower and higher than 3.6 mM resulted in lower enzymatic activity (data not shown). Concentrations of 20 μM substrate and 3.6 mM CHAPS were used for most of the further studies of the phospholipid acyl hydrolase. Although this PC concentration was far from substrate saturation, it was necessary to limit the amount of substrate added due to the great difficulties in preparing large quantities of [^{14}C]ricinoleoyl- and [^{14}C]vernoleoyl-PC. It should thus be noted that the specific activities of the acyl hydrolases presented in this paper are below the V_{max}.

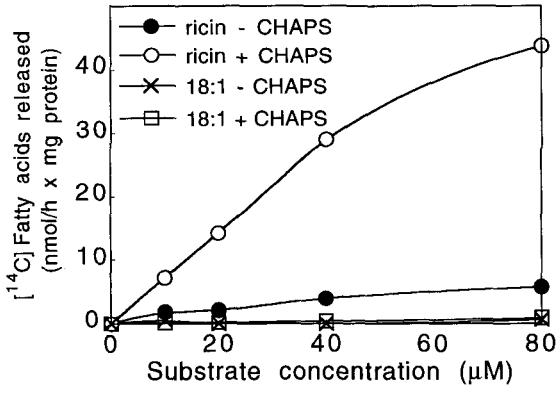

Figure 1. The effects of substrate concentration and the detergent CHAPS on microsomal *sn*-2-acyl-PC hydrolysis. *R. communis* microsomes were incubated with *sn*-2-[^{14}C]ricinoleoyl-PC without CHAPS (●) or with 3.6 mM CHAPS (○) and with *sn*-2-[^{14}C]18:1-PC without CHAPS (×) or with CHAPS (□), for 120 min at 30°C. The reaction mixture consisted of 2 to 16 nmol of [^{14}C]PC, microsomal membranes (equivalent to 100 μg of microsomal protein) in 0.1 M potassium phosphate buffer, pH 7.2, in a final volume of 0.2 mL. The data are means from two incubations.

Acyl Dependence of Microsomal *sn*-2-Acyl-PC Hydrolysis

The time course of acyl hydrolysis of different *sn*-2-[^{14}C]acyl-PC species incubated with microsomes prepared from *R. communis* and *U. glabra* developing seeds are shown in Figure 2. Both microsomal preparations were essentially unable to catalyze the hydrolysis of [^{14}C]oleoyl-PC and [^{14}C]vernoleoyl-PC, whereas [^{14}C]ricinoleoyl-PC and [^{14}C]caproyl-PC were deacylated at high rates by *R. communis* and *U. glabra* microsomes, respectively. The hydrolysis of caproyl and ricinoleoyl groups by *U. glabra*

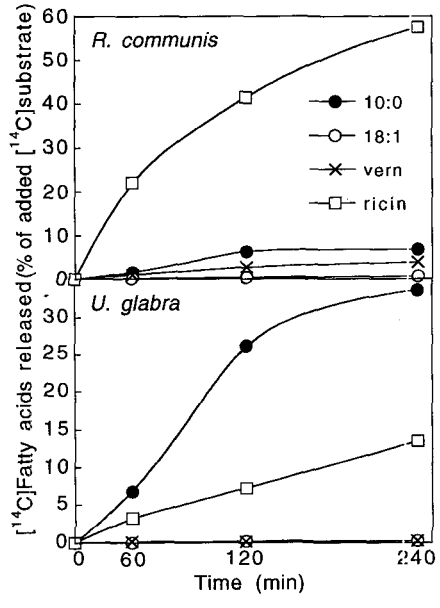

Figure 2. Time-course studies of *sn*-2-acyl-PC hydrolysis in microsomes from *R. communis* and *U. glabra*. PC substrates used: *sn*-2-[^{14}C]10:0 (●); *sn*-2-[^{14}C]18:1 (○); *sn*-2-[^{14}C]vernoleate (×); *sn*-2-[^{14}C]ricinoleate (□). Standard assay conditions used were as described in "Materials and Methods." The data are means from two incubations.

membranes appeared linear with time for up to 120 min of incubation, whereas the ricinoleoyl groups were removed in a nonlinear fashion in assays with *R. communis* membranes.

Added [^{14}C]oleoyl-PC substrate was diluted with endogenous oleate and other common acyl groups in the membranes, whereas the dilution of the [^{14}C]PC with uncommon acyl groups was much less, since these acids are present only in low amounts in the membranes (see Table I). Although the differences in release of ^{14}C-label between PC substrates with oleate and uncommon fatty acids in the above experiments demonstrate a selectivity for uncommon acyl groups in PC, they might not reflect differences in the hydrolysis of the absolute amounts of the different acyl groups in the membranes. To determine the extent of total acyl hydrolysis in the membranes, the quantity and quality of the free fatty acid pool were analyzed before and after incubations of microsomal membranes.

Table II shows the changes in amounts of acyl groups in free fatty acids and in PC after incubations (2 h) of *U. glabra* membranes without and with added *sn*-1–16:0-*sn*-2–18:1-PC or *sn*-1–16:0-*sn*-2–10:0-PC substrates. The formation of free fatty acid without added PC substrate was low, with only a slight but significant increase in palmitic acid. Addition of 8, 16, and 32 nmol of *sn*-2–18:1-PC did not further increase the total amount of free fatty acids released. Significantly elevated levels of free linoleic acids were noted, but this was not matched by a decrease of that acid in PC. Addition of *sn*-2–10:0-PC caused a concentration-dependent increase in free capric acid with a corresponding decrease in the amount of this acid in PC. The decrease in the amount of palmitoyl groups in PC was less than the decrease in caproyl groups. Since palmitate was present in position *sn*-1 of the added 10:0-PC substrate, a similar decrease of palmitate and the uncommon fatty acid in PC would have been expected. However, as demonstrated in experiments reported below, these membranes contained active 16:0-LPC transacylase activity, which efficiently reincorporated the 16:0-LPC into PC (see Fig. 5III, A). This reaction could entirely explain the observed nonstochiometric removal of palmitate and caproate from PC. There was also a *sn*-2–10:0-PC-dependent increase in free palmitic acid, which was less than half of the 10:0 release. The release of free palmitate is likely to be due to the activity of an LPC acyl hydrolase, although this enzyme activity was quite low in *U. glabra* membranes when measured with added 16:0-LPC (see Fig. 5II, A).

In conclusion, the data in Table II clearly show that, in *U. glabra* membranes, total acyl hydrolase activities toward membrane lipids with common fatty acids were, at the most, 10 to 20% of the release of fatty acids from added *sn*-2–10:0-PC, and that 18:1-PC did not serve to a significant extent as substrate for the acyl hydrolase. Analysis of the amounts of acyl groups in free fatty acids and PC from incubations of microsomal membranes of *R. communis* with and without added *sn*-2–18:1-PC and *sn*-2-ricinoleoyl-PC were also performed. The results showed, similar to the *U. glabra* membranes, that added PC with the uncommon fatty acid (in this case ricinoleoyl-PC) was hydrolyzed 5 to 10 times faster than all other acyl groups in the membranes, and that no significant release of acyl groups could be monitored from 18:1-PC (data not shown). Thus, the microsomal acyl hydrolases had both high specificities and selectivities for PC with the uncommon acyl groups. GC chromatograms of the fatty acid profile of PC isolated before and after an incubation (2 h) of *R. communis* membranes with 32 nmol of exogenous ricinoleoyl-PC illustrates the selective removal of the ricinoleoyl groups from this lipid (Fig. 3).

When the release of free fatty acids without added PC was monitored in *B. napus* membranes, we found that these preparations, in contrast to the *U. glabra* and *R. communis* microsomal membranes, had considerable acyl hydrolase activities toward common fatty acids. Since the release of

Table II. *Changes in amounts of acyl groups in free fatty acids (FFA) and PC in 2-h incubations of microsomal preparations of developing U. glabra seeds with and without exogenous PC*

Acyl Group	Changes in Acyl Groups (nmol)													
	Substrate added													
	sn-1–16:0-*sn*-2–18:1-PC (nmol)								*sn*-1–16:0-*sn*-2–10:0-PC (nmol)					
	None		8		16		32		8		16		32	
	FFA	PC	FFA	PC	FFA	PC	FFA	PC	FFA	PC	FFA	PC	FFA	PC
10:0	0	0	0	0	0	0	0	0	+3.0[a]	−2.8[a]	+3.7[a]	−3.9[a]	+4.9[a]	−5.9[a]
14:0	0.0	−0.1	0	0	0	−0.1	0	+0.2[a]	+0.1	0	+0.1	0	+0.2[a]	+0.1
16:0	+0.5[a]	−0.6	+0.4[a]	+1.0	+0.5[a]	+0.4	+0.7[a]	+2.2	+1.3[a]	−1.2	+1.7[a]	−2.8[a]	+2.8[a]	−2.1
18:0	0	+0.2	0	−0.1	0	+0.1	+0.1	+0.2	+0.2[a]	0.0	+0.1[a]	+0.1	+0.2[a]	+0.2
18:1	0.1	+0.3	+0.2	+1.2	+0.2	+0.3	+0.4[a]	+1.0	+0.2	−0.3	+0.1	−0.8	+0.1	−0.1
18:2	+0.1	+1.6	+0.5[a]	+1.6	+0.5[a]	+3.1	+0.5[a]	+0.5	+0.5[a]	−0.1	+0.3[a]	−2.3	+0.2	+1.1
18:3	+0.1	−0.2	+0.2	0.0	+0.2	0.0	+0.2	0	+0.2	+0.4	+0.2	0	+0.2	+0.4
Total FFA formed	+0.8		+1.3		+1.4		+1.9		+5.5[b]		+6.2[b]		+8.6[b]	

[a] Denotes significant differences between amounts of a particular acyl group before incubation (n = 19) and after incubation (2 h) without or with added PC at specified concentration (n = 3) in a mean difference two-sided test (α = 5). [b] Denotes significant differences between the increase in total FFA without added substrate (n = 3) and with added PC at specified concentration (n = 3) in a mean difference two-sided test (α = 5).

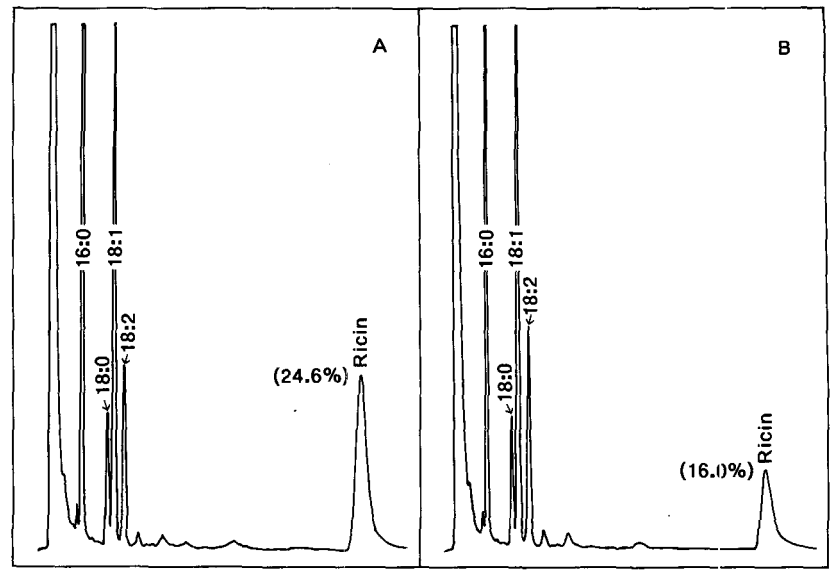

Figure 3. GC chromatograms of the fatty acid profile (methyl esters) of PC isolated at zero time (A) and after 2-h incubations (B) of microsomal membranes of developing *R. communis* endosperm and 32 nmol of exogenous *sn*-1–16:0-*sn*-2-ricinoleoyl-PC. The relative proportions (mol%) of ricinoleate for each incubation is given in parentheses.

free fatty acids from endogenous lipids in *B. napus* membranes would mask contributions made from the release of acyl groups from added PC with common acyl groups, it was not possible with this method to determine the extent to which these acyl hydrolases could act on exogenous PC substrates.

The release of [^{14}C]fatty acids from different *sn*-2-[^{14}C]acyl-PC substrates incubated for 120 min with microsomal preparations from the oil storage tissues of the different plants is presented in Figure 4, A to F. The hydrolysis of *sn*-2[^{14}C]palmitoyl-PC was low but significant in all membranes (Fig. 4A). The hydrolysis of [^{14}C]oleoyl-PC was low but significant in rape membranes, but barely above the detection limit in the other membranes (Fig. 4B). Vernoleoyl-PC was efficiently hydrolyzed by the *E. lagascae* membranes but hardly utilized at all by the enzymes in *U. glabra* and *R. communis* membranes (Fig. 4C). Ricinoleoyl-PC was hydrolyzed at a different extent by the different membranes, with *R. communis* membranes having the highest activity (46% of the substrate hydrolyzed) and *U. glabra* having the lowest activity (6% of the substrate hydrolyzed) (Fig. 4D). The percentage hydrolysis of [^{14}C]caproyl-PC was only slightly higher than [^{14}C]16:0-PC in incubations with *B. napus* membranes, whereas membranes from *C. procumbens*, *U. glabra*, and *E. lagascae* hydrolyzed this substrate, on a percentage basis, 10- to 60-fold more efficiently than the 16:0-PC species (Fig. 4E). [^{14}C]Lauroyl-PC was hydrolyzed at a substantially higher extent than the common PC species by the *U. glabra* membranes, whereas the other membranes showed similar or only moderately elevated levels of hydrolysis with this substrate compared to the 16:0-PC substrates (Fig. 4F).

sn-1-Acyl-PC and *sn*-1-Acyl-LPC Hydrolysis and Transacylation

Different *sn*-1-[^{14}C]acyl-PC and *sn*-1-[^{14}C]acyl-LPC species were tested as substrates for the acyl hydrolase in microsomes from the four plant species (Fig. 5I, A–E). The *sn*-1-[^{14}C]16:0-PC and *sn*-1-[^{14}C]18:1-PC substrates were, like the *sn*-2 positional isomers, poor substrates (Fig. 5I, A and B). Similar to the acyl specificity toward the *sn*-2 position (Fig. 4C), *E. lagascae* membranes also showed high activities toward substrates containing vernoleoyl groups at the *sn*-1 position (Fig. 5I, C). The *sn*-1-[^{14}C]ricinoleoyl-PC was as efficient as substrate as the *sn*-2 ricinoleoyl-PC for the *R. communis* acyl hydrolases (compare Fig. 5I, D and Fig. 4D). However, since the *sn*-1-[^{14}C]ricinoleoyl-PC substrate contained a rather racemic positional distribution of radioactivity (only 53% of the radioactivity was in position *sn*-1), the contribution of each position in the acyl hydrolysis could not be established just by measuring the ^{14}C-free fatty acid released. Therefore, unhydrolyzed [^{14}C]PC (44% of added [^{14}C]PC) was reanalyzed for the positional distribution after incubation with *R. communis* membranes. The distribution of radioactivity between the different positions was not changed by incubation, which indicates that ricinoleoyl hydrolysis occurred at approximately the same rate in the *sn*-1 and the *sn*-2 positions. *sn*-1-[^{14}C]10:0-PC was a much less efficient substrate than the *sn*-2 positional isomer for the *U. glabra* and *C. procumbens* acyl hydrolase activities, whereas the reverse was the case in *R. communis* (compare data in Fig. 4E and Fig. 5I, E).

Contrary to the situation with the PC substrates, radioactive 16:0 and 18:1 were also hydrolyzed to a significant extent from the *sn*-1-[^{14}C]acyl-LPC (Fig. 5II, A and B). *sn*-1-[^{14}C]acyl-LPC with vernoleoyl or ricinoleoyl groups was hydrolyzed with activities comparable to those of corresponding *sn*-1-acyl-PC substrates by the *E. lagascae* and *R. communis* membranes, respectively (Fig. 5II, C and D). The *C. procumbens* membranes hydrolyzed the *sn*-1-[^{14}C]10:0-LPC at a considerably higher rate than the *sn*-1-[^{14}C]10:0-PC substrate (Fig. 5II, E).

In addition to acyl hydrolysis, considerable radioactivity from *sn*-1-[^{14}C]acyl-LPC was also incorporated into PC,

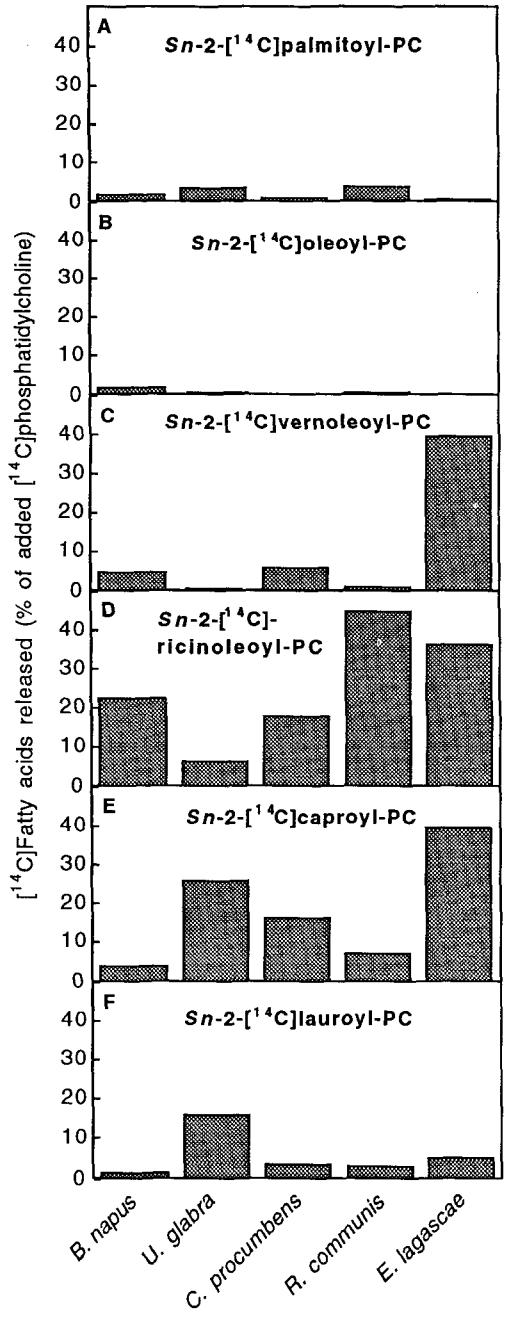

Figure 4. Acyl dependence of *sn*-2-acyl-PC hydrolysis. Six different *sn*-2-[^{14}C]acyl-PC species (A–F) were compared as substrates for *sn*-2-acyl hydrolysis in microsomes from developing embryos/endosperms of five different plants. The release of ^{14}C-fatty acids was determined after 120 min of incubation at 30°C. Assays were performed under standard conditions, as described in "Materials and Methods." The data are means from two incubations.

indicating transacylation activities (Fig. 5III). The acyl specificities of the transacylation were distinctly different from those of acyl hydrolase activities. The highest transacylase activities were found with 16:0- and 18:1-LPC (Fig. 5III).

Comparison between *sn*-2-Acyl-Hydrolysis of PC, PE, and PA

The extent of acyl hydrolysis of *sn*-2-[^{14}C]acyl-PA and *sn*-2-[^{14}C]10:0-PE were compared with the hydrolysis of *sn*-2-[^{14}C]acyl-PC in incubations with *U. glabra*, *C. procumbens*, *E. lagascae*, and *R. communis* membranes (Fig. 6). *sn-*

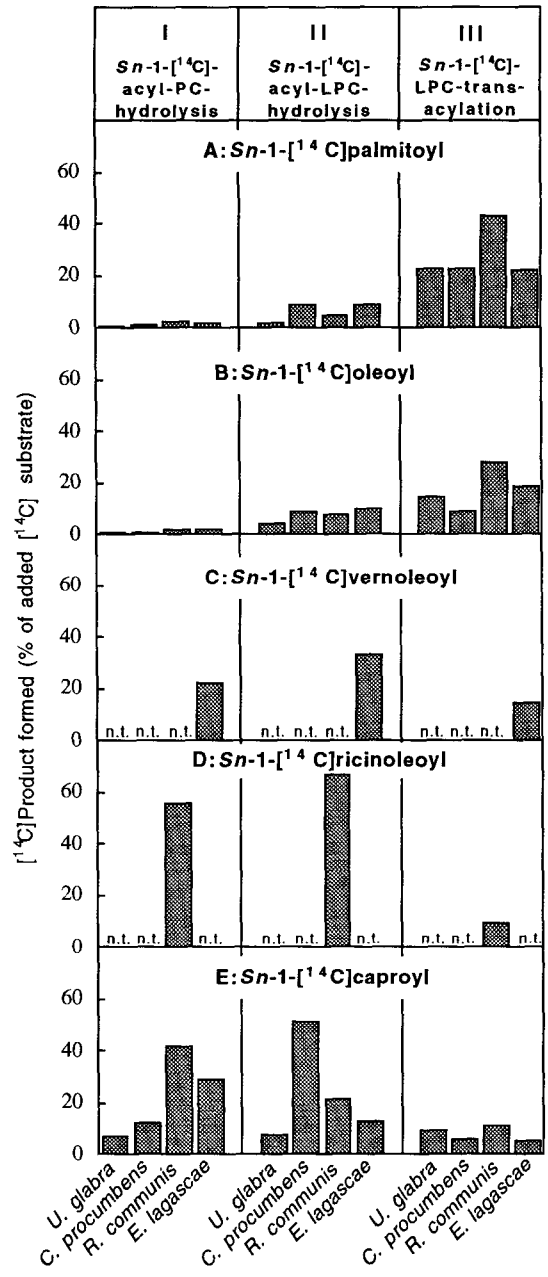

Figure 5. Acyl dependence of the *sn*-1-acyl-PC hydrolysis (I), *sn*-1-acyl-LPC hydrolysis (II), and LPC transacylation (III) in microsomal preparations from developing embryos/endosperms of four different plant species. The release of ^{14}C-fatty acids from [^{14}C]PC or [^{14}C]LPC and the formation of [^{14}C]PC from [^{14}C]LPC were determined after 2 h of incubation at 30°C. Assays were performed under standard conditions as described in "Materials and Methods." n.t., Not tested. The data are means from two incubations.

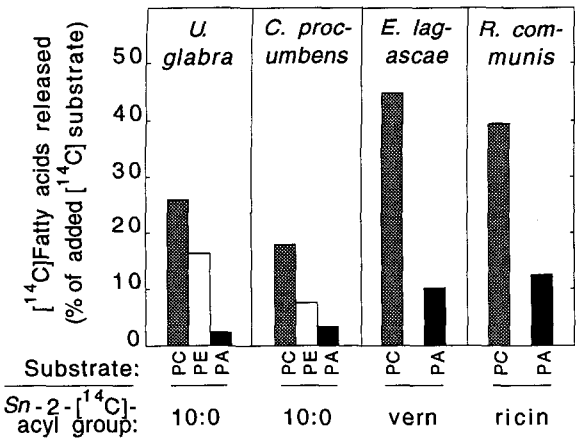

Figure 6. Comparison of the *sn*-2-acyl hydrolysis of PC, PE, and PA by microsomal preparations from embryos/endosperms of four different plant species. The release of ^{14}C-fatty acids from the *sn*-2-[^{14}C]acyl-phospholipids was determined after incubation for 120 min at 30°C. Assays were performed under standard conditions as described in "Materials and Methods." vern, Vernoleate; ricin, ricinoleate. The data are means from two incubations.

2-[^{14}C]10:0-PE was hydrolyzed at an extent of 41 to 77% of that of the *sn*-2-[^{14}C]10:0-PC substrate. The *sn*-2-[^{14}C]acyl-PA containing radioactive 10:0, vernoleoyl, or ricinoleoyl groups were hydrolyzed at 9 to 32% of that of corresponding *sn*-2-[^{14}C]acyl-PC molecular species.

The Effects of EGTA and Ca^{2+} on Microsomal Acyl Hydrolases and Phospholipase D Activities

The effects of addition of EGTA or Ca^{2+} on the acyl hydrolysis of the different [^{14}C]acyl PC substrates by microsomal preparations from different plants were generally small (data not shown). When effects were observed, both additions of Ca^{2+} (5 mM) or EGTA (5 mM) were inhibitory to fatty acid release compared to incubations without any of these compounds. In addition to ^{14}C-fatty acid release, [^{14}C]PA was also formed in incubations of [^{14}C]acyl-PC substrates with microsomal membranes, indicating activity of a phospholipase D. Contrary to the acyl hydrolase, the phospholipase D activities were strongly stimulated by addition of Ca^{2+} and totally, or nearly totally, inhibited by EGTA (data not shown).

Acyl Specificities of Microsomal Phospholipase D Activities

Phospholipase D activities in microsomal preparations from embryos and endosperms were measured with different *sn*-2-[^{14}C]acyl-PC substrates in 2-h incubations in the presence of Ca^{2+} (Fig. 7). The activities were somewhat dependent on acyl quality, with higher activities with medium-chain or ricinoleoyl substrates than with PC with common or vernoleoyl acyl groups. Contrary to the acyl hydrolase activities, however, the differences in phospholipase activity between common-, medium-chain- and oxygenated-acyl substrates were relatively small and membranes from all plant species showed the same pattern of acyl specificities. However, the absolute amount of PA produced differed between membranes from different plant species.

DISCUSSION

Plant microsomal acyl hydrolase activities toward PC were greatly influenced by acyl quality. Not only hydroxy-

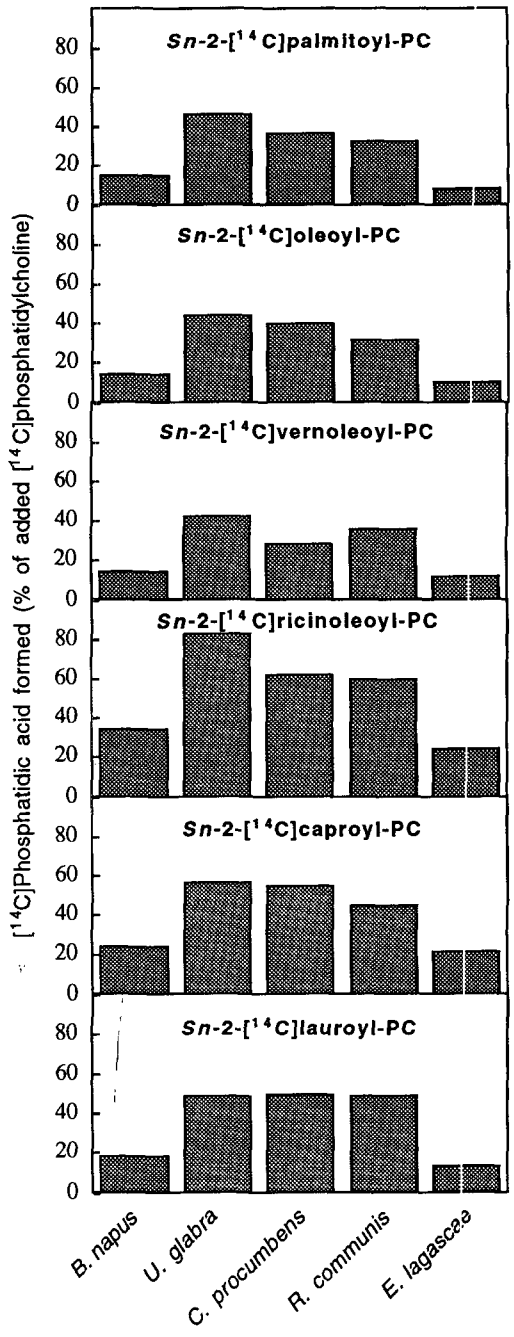

Figure 7. Acyl dependence of microsomal phospholipase D activities. The production of [^{14}C]PA was determined after incubation of exogenous *sn*-2-[^{14}C]acyl-PC with microsomal preparations of endosperms/embryos of different plant species. Assays were performed as described in "Materials and Methods," but in the presence of 5 mM $CaCl_2$. The data are means from two incubations.

lated and epoxidated, but also medium-chain acyl groups, could be hydrolyzed at substantial rates by membranes from some of the plant tissues. Although the plant membranes also contained some acyl hydrolase activities toward common acyl groups, these activities were very minor in *U. glabra* and *R. communis* membranes and no significant hydrolysis of 18:1-PC substrate was observed. Such pronounced acyl selectivities and specificities of phospholipid deacylating enzymes have, to our knowledge, only been demonstrated earlier for intracellular arachidonoyl-specific phospholipase A_2 in animal cells (Clark et al., 1991). The question arises whether the acyl specificities and selectivities for the uncommon acyl groups are due to the physical properties of the substrate that facilitate its accessibility to the enzyme, as is proposed to be the mechanism behind the somewhat more rapid removal of oxidated acyl groups by phospholipases A_2 in animal membranes (Sevanian et al., 1988), or if it is an inherent selectivity of the enzymes. If substrate accessibility would be the only cause for acyl selectivity, the phospholipases from different plant species would be expected to have similar relative activities toward the different acyl groups. This was the case for the microsomal phospholipase D activity in this study. The phospholipid acyl hydrolases, on the other hand, showed sometimes radically different acyl selectivities in the membranes from the different plant species examined. It could, however, be argued that a particular acyl group could cause different physical effects on the different membrane types. This is unlikely to be the cause for the differences in the phospholipid acyl hydrolase selectivities in plants. For example, *sn*-2-ricinoleoyl-PC was a very good substrate for acyl hydrolases in both *R. communis* and *E. lagascae* membranes. *sn*-2-vernoleoyl-PC was as effective as *sn*-2-ricinoleyl-PC for *E. lagascae* microsomes, but the epoxidized substrate was hardly hydrolyzed at all by the *R. communis* membranes. It is thus difficult to explain the acyl hydrolase activities only as a consequence of alteration in membrane structures caused by the substrates, and it is more likely due to acyl specificities and selectivities exerted by the enzymes themselves.

Plant tissues accumulating triacylglycerols rich in capric (decanoic) groups (i.e. developing embryos of *C. procumbens* and *U. glabra*) also had acyl hydrolases with high activity toward PC with this acyl group. Similarly, *E. lagascae* endosperms and *R. communis* embryos, which accumulate triacylglycerols with vernoleate and ricinoleate, respectively, had microsomal phospholipases that removed their respective oxygenated acyl group from the phospholipids. The physiological significance of phospholipid acyl hydrolases with high specificities for ricinoleoyl- and vernoleoyl-containing substrates in *R. communis* and *E. lagascae* endosperms is obvious. *R. communis* and *E. lagascae* endosperms accumulate ricinoleate and vernoleate, respectively, as their dominating acyl constituent in the triacylglycerols, and the synthesis of these acids occur via hydroxylation and epoxidation of precursor acyl groups esterified in phospholipids (Bafor et al., 1991, 1993). Thus, the phospholipases could remove the newly synthesized oxygenated fatty acids from the phospholipids for subsequent channeling into triacylglycerols (Bafor et al., 1991). It can be assumed that the high activity toward 10:0-PC seen in microsomal membranes from tissues that accumulate 10:0-rich triacylglycerols can be functionally related to the removal of 10:0 groups that enter the phosholipids during active triacylglycerol biosynthesis in these tissues. An accumulation of high amounts of epoxy, hydroxy, or capric acyl groups in membrane lipids would certainly be deleterious to membrane structure and function. In tissues not accumulating oxygenated acyl groups, the presence of phospholipases with high activity toward such groups might serve as parts of a restoration system for membrane integrity after oxidative damage to the phospholipids, as suggested earlier (Banas et al., 1992). It could also be hypothesized that these enzymes could be involved in releasing oxygenated fatty acids as precursors for the synthesis of fatty acid-based plant hormones (Leshem, 1987), and pathogen defense substances (Farmer and Ryan, 1992). The synthesis of these substances would then not be initiated by the release of a polyunsaturated fatty acid by a phospholipase but by an oxygenated metabolite of this acid. With present knowledge, we cannot suggest any physiological function for phospholipid acyl hydrolases with high activity toward medium-chain acyl groups in membranes from tissues that do not accumulate such acids.

The phospholipid acyl hydrolases specific for unusual acyl groups might also attack PA and therefore could be counterproductive for synthesis of triacylglycerols containing such acids. However, it should be noted that PA was a considerably less-efficient substrate than PC for the acyl hydrolases. Also, the PA pool is very minor (2–5%) compared to the total phospholipid pool in developing oil seeds (S. Stymne, unpublished results). Thus, the contribution of PA in the release of uncommon acyl groups from phospholipids could be expected to be neglible.

The production of LPC from PC by the action of an acyl hydrolase necessitates a rapid metabolism of the lysophospholipids, which otherwise would damage membrane integrity. Present work demonstrates that plant membranes can either hydrolyze the LPC or transacylate it into PC. The transacylation reaction occurs preferentially with lyso-PC containing common membrane fatty acids and thus seems to be catalyzed by enzymes other than the acyl hydrolysis.

Similar acyl selectivities were obtained for the *sn*-1 and *sn*-2 positions of PC as well as for *sn*-1-LPC in the hydrolysis of epoxy and hydroxy acyl groups. However, the positional localization of the medium-chain acyl groups had great influence on the activities in a particular membrane. The isolation and further characterization of these phospholipid acyl hydrolases in developing seeds will yield more precise information of their number, nature, and relationship with already characterized phospholipases and acyl hydrolases. Such work is now in progress in our laboratory.

ACKNOWLEDGMENTS

Dr. R. Kleiman, U.S. Department of Agriculture (Peoria, IL); Dr. J.T.P. Dereksen, ATO-DLO, Wageningen, The Netherlands; and

Mr. M Ivarsson, Uppsala Botanical Garden, Sweden, are gratefully acknowledged for providing seed materials for cultivation.

Received September 23, 1994; accepted November 23, 1994.

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