FEBS Letters 464 (1999) 80–84 FEBS 23111

One of the origins of plasma membrane phosphatidylserine in plant cells is a local synthesis by a serine exchange activity

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Received 3 November 1999

Edited by Guido Tettamanti

Abstract In plant cells, as in animal cells, the endoplasmic reticulum (ER) is considered to be the major site of phospholipid synthesis, and it has been shown that phosphatidylserine (PS) reaches the plasma membrane via the vesicular ER-Golgi-plasma membrane pathway in leek cells. However, it has never been determined whether the plasma membrane of leek cells is able to synthesize PS. We have analyzed the distribution of PS synthesizing enzymes along the vesicular pathway. In ER, Golgi and plasma membrane fractions isolated from leek cells, we have measured the activity of the two biosynthetic pathways leading to the synthesis of PS, i.e. serine exchange and CTP cytidylyltransferase plus PS synthase. We have found a high serine exchange activity in the plasma membrane fraction, and then determined that this membrane is able to synthesize both long chain fatty acid- and very long chain fatty acid-containing PS. Therefore, the PS in the plasma membrane of leek cells has two different origins: the intracellular vesicular pathway from the ER and a local synthesis in the plasma membrane.

Key words: Plant cell; Plasma membrane; Phosphatidylserine synthesis; Serine exchange activity

1. Introduction

In plant cells, the endoplasmic reticulum (ER) is the major site of phospholipid synthesis [1] and these molecules are then transferred to the plasma membrane [2–5]. Recent studies devoted to their transport have shown that the different phospholipid species do not follow the same route from the ER to the plasma membrane. Low temperatures and treatment with monensin have shown that phosphatidylserine (PS), sterols and very long chain fatty acid (VLCFA)-containing phosphatidylcholine (PC) and phosphatidylethanolamine (PE) exclusively follow the vesicular ER-Golgi-plasma membrane pathway in leek cells [2–5]. In vivo pulse-chase experiments with [1-^{14}C]acetate clearly indicated that these lipids were transferred from the ER to the plasma membrane with similar kinetics [2,4,5].

Although the ER appears to be the major source of phospholipids in the plasma membrane, the possibility of the latter's being able to synthesize some of them locally and especially PS, a phospholipid which is enriched in this membrane [6], cannot be excluded. In plant cells, the major pathway for the synthesis of PS is uncertain, two biosynthetic pathways could be operational [7]. A CMP-phosphatidic acid (PA): L-serine 3-phosphatidyltransferase (PS synthase) has recently been cloned in wheat (*Triticum aestivum*) [8]. The other pathway (i.e. the activity of base exchange) is also found in various plant cells [1].

It has been shown that the plasma membrane of rat liver is capable of synthesizing certain phospholipids by base exchange reactions [9,10]. Therefore, in order to determine whether the plasma membrane of plant cells is able to synthesize PS, we have measured the activity of the two biosynthetic pathways leading to the synthesis of PS in plasma membrane fractions isolated from leek cells and compared it with the activities found in ER and Golgi membrane fractions.

We have found a high serine exchange activity to be present in the plasma membrane fraction in vitro, and have tried to determine which PS species are synthesized within the plasma membrane by this enzyme activity.

2. Materials and methods

2.1. Plant material and chemicals

Leek (*Allium porrum* L.) seeds were purchased from Vilmorin (France). They were stored overnight at 4°C before being hydrated with distilled water for 2 h. Then, they were allowed to germinate in the dark for 7 days at 22–24°C as already described [11].

All the chemicals were purchased from Sigma (St. Louis, MO, USA). [1-^{14}C]Acetate (53.9 Ci/mol) was obtained from CEA (Saclay, France) and Amersham France. L-[^{3}H(G)]Serine (19.7 Ci/mmol) was from NEN (Boston, MA, USA).

2.2. Isolation of ER, Golgi and plasma membrane fractions

Crude ER, Golgi and plasma membrane fractions were obtained on sucrose density gradients as reported earlier [12]. Briefly, leek seedlings were homogenized in a mortar in the presence of 10 mM KH_2PO_4 (pH 8.2) with 0.5 M sorbitol, 5% (w/v) PVP 40, 0.5% (w/v) BSA, 2 mM salicylhydroxamic acid and 1 mM PMSF. The homogenate was then filtered through two layers of Miracloth (Calbiochem) and subjected to differential centrifugations at $1000\times g_{max}$ for 10 min, $10\,000\times g_{max}$ for 10 min and $150\,000\times g_{max}$ for 60 min. The resulting microsomal pellet ($150\,000\times g_{max}$) was resuspended in

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Abbreviations: HPTLC, high performance thin layer chromatography; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; VLCFA, very long chain fatty acids (more than 18 carbon atoms)

PII: S0014-5793(99)01682-8

10 mM KH_2PO_4 and 0.5 M sorbitol (pH 7.8). Half of the membrane suspension was loaded onto a discontinuous sucrose density gradient consisting of 2.5 ml of 37% (w/v), 3.5 ml of 25% and 3.5 ml of 18%. After centrifugation at $150\,000\times g_{max}$ for 150 min, membranes at the 18%/25% (crude ER fraction) and 25%/37% (crude Golgi fraction) sucrose interfaces were collected, diluted with 10 mM KH_2PO_4 (pH 7.8) containing 0.5 M sorbitol and centrifuged at $150\,000\times g_{max}$ for 60 min. The pellets obtained and the crude plasma membrane fraction (corresponding to the pellet in the gradients obtained after the centrifugation of the microsomal pellet at $150\,000\times g_{max}$ for 150 min) were resuspended in the appropriate buffers for enzyme assays.

ER, Golgi and plasma membranes were then further purified by phase partitioning (this study).

To purify the ER and Golgi membranes by removing most of the contaminating plastids, plasma membranes and tonoplast according to Morré et al. [13], the crude ER and Golgi pellets were resuspended in 0.5 ml 0.25 M sucrose containing 5 mM KH_2PO_4 (pH 6.8) and applied to 25 ml of a two-polymer-phase system consisting of 5.9% (w/w) polyethylene glycol (PEG 4000) and 5.9% (w/w) dextran T-500 (Sigma), which were thoroughly mixed by vigorously inverting the tubes 40 times, and the phases resolved by low speed (10 min, $500\times g_{max}$) centrifugation. The lower phases, containing the ER- and Golgi-enriched fractions, were collected without disturbing the interfaces, sedimented by centrifugation for 60 min at $150\,000\times g_{max}$ and resuspended in the appropriate buffers for enzyme assays.

Purified plasma membranes were further obtained by phase partitioning using PEG 4000 and dextran T-500 as previously reported [12]. The other half of the microsomal suspension or the crude plasma membrane pellet from sucrose gradients were mixed with a PEG/dextran mixture in 0.5 M sorbitol containing 10 mM KH_2PO_4 (pH 7.8) and 40 mM NaCl to obtain final PEG 4000 and dextran T-500 concentrations of 6.0% (w/w). The solution (final volume: 28 ml) was mixed by vigorously inverting the tubes 40 times and centrifuged for 15 min at $1000\times g_{max}$. The PEG-enriched upper phase (12 ml) was recovered without disturbing the interface. Membranes were then recovered after centrifugation at $150\,000\times g_{max}$ for 60 min and resuspended in the appropriate buffer for enzyme assays.

Specific membrane compartments were identified by assays for the following markers: ER, NADPH-cytochrome *c* reductase and cholinephosphotransferase; Golgi apparatus, glucuronyltransferase; plasma membrane, glucan synthetase II and K^+-stimulated Mg^{2+} ATPase [2,11,12]. A low contamination of ER, Golgi and plasma membrane fractions by mitochondrial and plastid membranes was observed [12].

Protein concentrations were determined by the method of Bradford [14] using BSA as a standard. For electron microscopy, membrane fractions purified by phase partitioning were fixed at 4°C by recovering the pellets with a fixative solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.5. Fixed pellets were then washed and post-fixed in 1% osmium tetroxide in the same buffer. After washing with distilled water, the samples were treated in an aqueous solution of 1% uranyl acetate for 30 min at room temperature, washed again, dehydrated, and epon-embedded. Each pellet was divided into several blocks that were cut into thin sections. The section plane was random but constant in thickness. Sections were contrasted with a uranyl acetate solution, and then with lead citrate. They were observed and photographed using a Philips EM 210 electron microscope.

2.3. Assays for CTP cytidylyltransferase, PS synthase and serine exchange enzyme

Enzyme assay conditions for the ER of leek cells were derived from those already performed on the ER of other plant systems [1].

The assay of CTP cytidylyltransferase was as follows: membranes (50 μg protein) were incubated for 5 min at 30°C in 10 mM MES, pH 6.5, with 10 mM β-mercaptoethanol, 7.5 mM $MgCl_2$ and 7.5 mM $MnSO_4$. Then, 500 μM of CTP was added and the membranes were further incubated in a stirring rod for 60 min at 30°C in a final volume of 50 μl. Membranes were centrifuged at $150\,000\times g_{max}$ for 15 min (Hitachi, Himac CS 100) and the pellets resuspended in 0.05 M Tris, pH 8.5. PS synthase was then measured as follows: membranes (10 μg protein) were incubated for 5 min at 30°C with 10 mM β-mercaptoethanol, 3 mM $MgCl_2$ and 2 mM $MnCl_2$. The reaction was then initiated by the addition of 2 mM of L-serine containing 7 μCi of L-[^{3}H(G)]serine and the membranes were incubated in a stirring rod for 30 min at 30°C in a final volume of 50 μl.

PS synthesis by the serine exchange enzyme was measured as indicated below. Membranes (10 μg protein) were incubated under shaking for 5 min at 30°C in 0.1 M HEPES, pH 7.8, containing 10 mM of β-mercaptoethanol and 10 mM $CaCl_2$. The reaction was then initiated by the addition of 2 mM of L-serine containing 7 μCi of L-[^{3}H(G)]serine, and the membranes were incubated in a stirring rod for 30 min at 30°C in a final volume of 50 μl.

The experimental conditions determined for the ER were then tested with the Golgi and plasma membrane fractions.

The reactions were stopped by adding 500 μl of chloroform/methanol (2:1, v/v). Newly synthesized PS was extracted by adding 500 μl of chloroform. The chloroformic PS extract was washed three times with 500 μl of distilled water to discard the radiolabelled serine, and the labelled PS was determined by liquid scintillation counting in a Packard 2000 CA scintillation counter as already described [2,11]. This synthesized PS was identified by chromatography on HPTLC plates and comigration with a PS standard (see below). The activities were expressed as nmol PS synthesized/mg protein/h.

2.4. In vivo labelling of phospholipids and PS synthesis by the isolated plasma membrane

For each experimental value, 10 batches of 20 leek seedlings (cut into 5–10 mm segments, including roots) were incubated in 0.2 ml of 20 μCi of [1-^{14}C]acetate (53.9 Ci/mol) for 4 h at 24°C [4,13]. After labelling, plasma membrane fractions were prepared by phase partitioning as indicated above. Half of the membranes were incubated for 60 min in the presence of serine, according to the experimental conditions described for the assay of serine exchange enzyme. The other half of the membranes were either kept on ice or incubated for 60 min with cofactors but in the absence of serine (control). Lipids of the plasma membrane fractions were then extracted by chloroform:methanol (2:1, v/v) for 30 min at room temperature. The lipid extracts were washed three times in distilled water. The solvent was evaporated and the lipids were resuspended in an appropriate volume of chloroform:methanol (2:1, v/v) according to procedures already described [2,11].

PS isolation was carried out on HPTLC plates (Merck 60 F_{254}) developed with methylacetate:*n*-propanol:chloroform:methanol: aqueous 0.25% (w/v) KCl (25:25:28:10:7, v/v) according to Heape et al. [15].

After identification by comparison with standards, PS was scraped off directly into vials and the radioactivity was determined by liquid scintillation counting in a Packard 2000 CA scintillation counter. PS radioactivity was also quantified with a phosphorimager from Molecular Dynamics-Pharmacia, this method allowing the recovery of the samples for fatty acid analysis. Both methods gave similar results.

2.5. Analysis of PS fatty acid content

Fatty acid methyl esters (FAME) were prepared by heating samples at 80°C in 1 ml of methanol, 2.5% H_2SO_4 (v/v) for 60 min in screw-capped tubes. After the addition of 1.5 ml hexane and 1.5 ml water, FAME were recovered from the organic phase by shaking, and a two-phase system was established by centrifugation ($1500\times g$, 10 min). FAME were analyzed by HPTLC on RP-18 F_{254S} reverse-phase plates eluted twice with acetonitrile/tetrahydrofuran (80/20, v/v). The radioactivity of the different fatty acids was quantified with a phosphorimager (Molecular Dynamics-Pharmacia), the identification of the fatty acids was achieved by comparison with standards.

3. Results and discussion

3.1. Isolation of highly enriched ER, Golgi and plasma membrane fractions from leek cells

ER, Golgi and plasma membrane fractions were first prepared from 7-day-old etiolated leek seedlings on density sucrose gradients and characterized by enzymatic markers as previously reported [2,11,12,16–18]. The corresponding fractions are noted ER(gradient), Golgi(gradient) and plasma membrane(gradient) in Table 1. The degree of purity achieved, according to marker enzyme activities, is of the same order of magnitude as previously reported [12].

These membranes were further purified by phase partitioning (this study) according to well-known procedures [12,13], and their degree of purity was also estimated by measuring

Table 1
Purity of ER, Golgi and plasma membrane fractions according to marker enzymes

	Proteins (mg)	NADPH-Cyt. *c* reductase		Choline phosphotransferase		Glucuronyl-transferase		Glucan synthetase II		K^+-stimulated Mg^{2+} ATPase	
		nmol/h/mg	enrichment factor	nmol/h/mg	enrichment factor	nmol/h/mg	enrichment factor	μmol/h/mg	enrichment factor	μmol Pi/h/mg	enrichment factor
Homogenate	180	0.40	1	0.44	1	0.044	1	0.054	1	0.051	1
ER (gradient)	4.4	2.90	7.3	4.5	10.2	0.059	1.34	0.016	0.29	0.006	0.12
Golgi (gradient)	8.7	0.45	1.1	2.25	5.1	0.138	3.14	0.041	0.76	0.029	0.57
Plasma membrane (gradient)	5.2	1.95	4.8	1.88	4.3	0.049	1.11	0.17	3.2	0.71	13.9
ER (phase partition)	0.07	–	–	11.6	26.4	0.065	1.48	nd	–	nd	–
Golgi (phase partition)	0.4	–	–	5.1	11.5	0.68	15.4	nd	–	nd	–
Plasma membrane (phase partition)	0.3	0.07	0.17	0.10	0.23	0.054	1.23	0.67	12.4	1.98	38.8

Activities of cholinephosphotransferase, NADPH-Cyt. *c* reductase, glucan synthetase II and K^+-stimulated Mg^{2+} ATPase were measured as previously described [2,9]. Glucuronyltransferase activity was assayed according to Hobbs et al. [17] and Baydoun and Brett [18]. Activity of succinate dehydrogenase in the membrane fractions was less than 1% of that found in the $12\,000 \times g$ pellet [11,14]. The membrane fractions obtained on sucrose gradients contained no more than 2% of total chlorophyll and carotenoids respectively [14]. Those molecules were not detected in the purified ER, Golgi and plasma membrane fractions obtained after phase partitioning. Enzyme activities were determined from three to six different fractions. nd: not detected. For the sake of clarity, S.D.s on marker enzyme activities are not included, the variations on the enrichment factors were 6–20%.

the various marker enzyme activities (Table 1). First, we must underline a low recovery of membrane material after phase partition but it is a well-known property of such purifications to be qualitative and not quantitative. However, the percentage of purified membranes recovered by these methods is relatively constant [2,12,13], and the reproducibility of their purity is indicated in Table 1.

A high increase of cholinephosphotransferase (enrichment factor of 26.4) was observed in the purified ER fraction, as expected. Interestingly, the purified Golgi fraction (enrichment factor of glucuronyltransferase of 15.4) also showed an increase in the cholinephosphotransferase activity (enrichment factor of 11.5), indicating that this organelle is somewhat capable of synthesizing PC as reported in other systems [7,19,20]. Both purified ER and Golgi fractions were devoid of any plasma membrane contaminant since no detectable glucan synthetase II and K^+-stimulated Mg^{2+} ATPase activities were found (Table 1).

The plasma membrane fraction was highly purified as shown by the enrichment factors of the plasma membrane markers glucan synthetase II (12.4) and K^+-stimulated Mg^{2+} ATPase (38.8), and the low values of the enrichment factors of the other markers (Table 1). It must be noted that the K^+-stimulated Mg^{2+} ATPase activity measured in the purified plasma membrane was 95% inhibited by vanadate [11,21].

Therefore, the ER, Golgi and plasma membrane fractions obtained after phase partitioning reached a sufficient degree of purity, enabling the study of PS synthesizing activities among them.

Fig. 1 shows the electron microscopy of the highly purified membrane fractions obtained after phase partitioning. It can be seen that the membrane materials recovered are mainly constituted by sealed vesiculated and tubular membranes.

3.2. PS synthesizing enzyme activities of the ER, Golgi and plasma membrane fractions isolated from leek cells

PS synthesizing enzyme activities were measured with enzyme assay conditions derived from those already performed on the ER of other plant systems [1]. We determined relative optimal assay conditions for the ER of leek cells, and then tested those conditions to measure enzyme activities in the Golgi and plasma membrane fractions. The first pathway of PS biosynthesis is constituted by CTP cytidylyltranferase and PS synthase. The first activity was performed using endogenous PA and exogenous CTP as substrates. Those conditions

Table 2
Enzyme activities of PS biosynthesis in leek membrane fractions

	CTP cytidylyltransferase+PS synthase		Ratio B/A	Serine exchange enzyme	
	nmol/h/mg (A)	enrichment factor		nmol/h/mg (B)	enrichment factor
Homogenate	0.12	1	1.8	0.22	1
ER (gradient)	0.83	6.92	0.9	0.75	3.4
Golgi (gradient)	0.33	2.75	2.5	0.82	3.7
Plasma membrane (gradient)	0.18	1.50	5.3	0.96	4.4
ER (phase partition)	0.87	7.25	1.2	1.06	4.8
Golgi (phase partition)	0.16	1.33	3.8	0.61	2.8
Plasma membrane (phase partition)	0.07	0.58	21.4	1.50	6.8

Enzyme activities were determined from at least three different fractions as detailed in Section 2. For the sake of clarity, S.D.s are omitted, the variations on the enrichment factors never exceeded 14%. PS synthase activity was measured in Tris buffer, whereas the serine exchange activity was measured in HEPES buffer. PS synthase activity could be contaminated by the serine exchange activity. However, we have determined that the serine exchange activity measured in Tris buffer is only 6–7% of that measured in HEPES buffer. The values of the serine exchange activities obtained in Tris buffer have been subtracted from the values obtained for PS synthase.

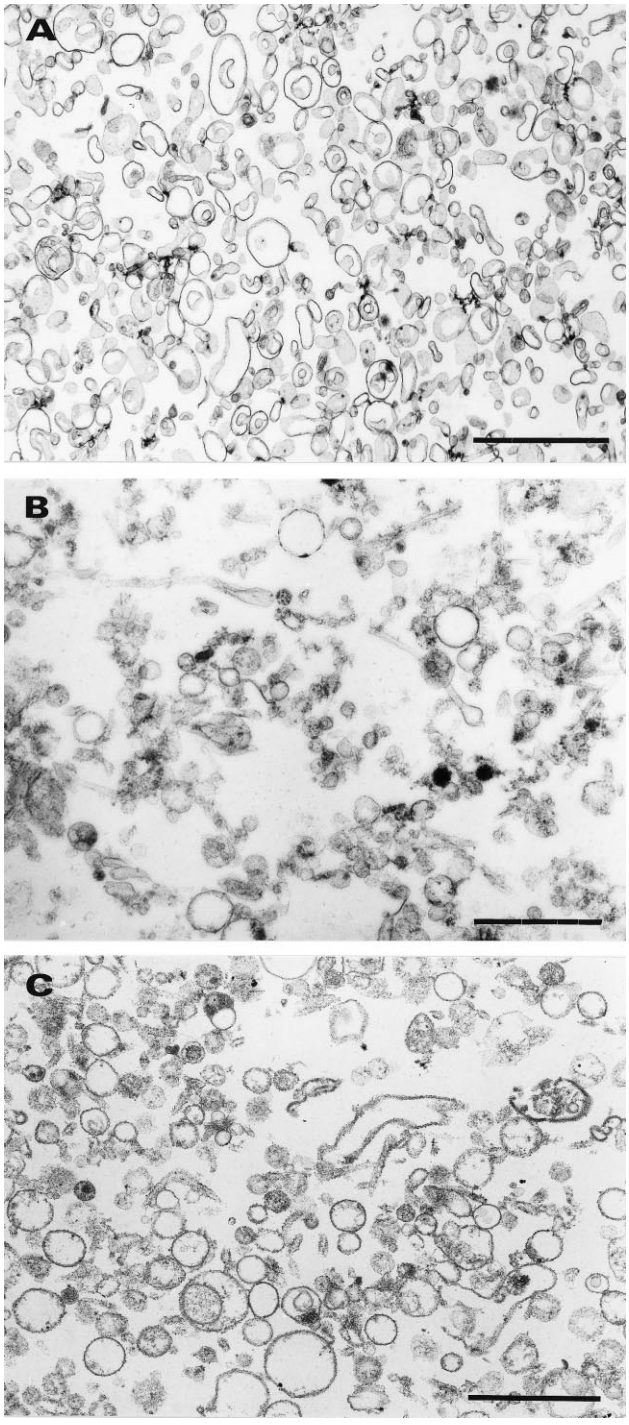

Fig. 1. Electron microscopy of the highly purified membrane fractions. Membrane fractions purified by phase partitioning were fixed and the samples were then treated as described in Section 2. A: Endoplasmic reticulum fraction. B: Golgi fraction. C: Plasma membrane fraction. Bar = 1 μm.

guarantee that the molecular species of CMP-PA synthesized are those that will normally be taken by PS synthase to form PS. In these conditions, we determined that the higher capability of synthesizing PS according to this biosynthetic pathway was found to be associated with the ER (Table 2). We checked that the plasma membrane synthesizes sufficient amounts of CMP-PA (0.97 nmol/mg/h) to sustain PS synthesis by PS synthase. CTP cytidylyltranferase activity (synthesizing CMP-PA) was measured using [^{3}H]CTP as substrate. Therefore, the absence of PS synthesis by this pathway reveals that there is no PS synthase activity in the plasma membrane. The capability of the latter to synthesize CMP-PA is probably required for PI resynthesis from phospholipase C and D products as suggested [22,23].

The serine exchange activity (the other pathway for PS synthesis) was more widely distributed (Table 2). This activity was significantly found in all membrane fractions, and a high capability of PS synthesis by this pathway was observed in the plasma membrane. The ratio of serine exchange activity to PS synthase was 21.4 for the plasma membrane and only 1.2 for the ER (Table 2).

It could be argued that we did not reach relative optimal assay conditions for the serine exchange activity in the plasma membrane fraction to compare with the ER. However, the PC and PE content of the plasma membrane (as mol% of total lipids) ranges from 30 to 50% whereas this content reaches 60–70% in the ER [6]. With potentially less substrate available for the serine exchange enzyme, the activity measured in the plasma membrane is higher than that in the ER. Moreover, whether we are close or not to relative optimal assay conditions for the plasma membrane activity will not change our results, which show that the plasma membrane has its own capability to synthesize PS, but will simply underestimate this activity. Therefore, we have unambiguously demonstrated the presence of a serine exchange activity in the plasma membrane of leek cells.

In conclusion, our results indicate that PS synthase activity is confined to endomembranes (mainly ER membranes and to a lesser extent Golgi membranes), and that the serine exchange activity is widely distributed with high activities in ER and plasma membranes.

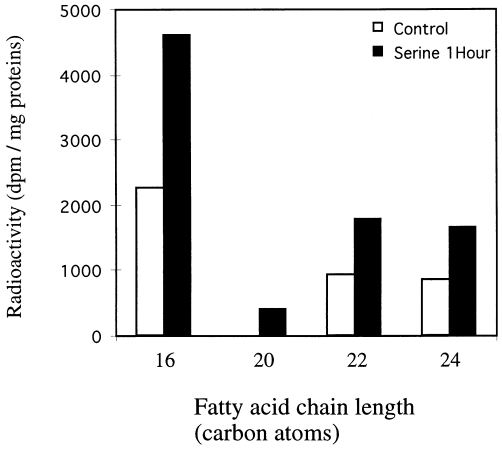

Fig. 2. Fatty acid composition of PS synthesized by the plasma membrane serine exchange activity. Experimental conditions for the serine exchange activity were as described in Section 2. The control corresponds to PS of the plasma membrane after a 4 h incubation period with acetate, without further incubation with serine. The other data correspond to PS of the plasma membrane after a 4 h incubation period with acetate, followed by a further incubation with serine and cofactors for 1 h. An increase in radioactivity and, therefore, synthesis of PS with fatty acyl chains having 16, 22 and 24 carbon atoms is observed.

3.3. Fatty acid content of PS synthesized by the plasma membrane serine exchange activity

For this purpose leek seedlings were incubated for 4 h at 24°C in the presence of [1-^{14}C]acetate (see Section 2). The plasma membrane was then isolated and incubated or not for 1 h in the presence of serine and cofactors required for the serine exchange activity. Lipids of the plasma membrane were then extracted and PS was purified by HPTLC. FAME of PS fatty acids were then analyzed by HPTLC on C-18 reverse phase plates (see Section 2). The radioactivity associated with the various fatty acids is shown in Fig. 2. The control corresponds to PS of the plasma membrane after the 4 h incubation period without a further incubation with serine (see Section 2). The other data correspond to PS of the plasma membrane after the 4 h incubation period followed by a further incubation with serine and cofactors for 1 h.

An increase of the radioactivity of fatty acyl chains having 16, 22 and 24 carbon atoms is observed inside the PS. These results indicate that the plasma membrane is able to synthesize long chain fatty acid- and very long chain fatty acid-containing PS by a serine exchange activity from endogenous PC and/or PE neosynthesized in the ER ([^{14}C]acetate-labelled) and delivered to the plasma membrane.

Concerning the VLCFA-containing phospholipids, we have demonstrated earlier that PC, PE and PS synthesized in the ER are then transferred to the plasma membrane by the vesicular pathway [2,4,6,13]. Therefore, the VLCFA-containing PS in the plasma membrane has two different origins: the intracellular vesicular pathway from the ER and a local synthesis from VLCFA-containing PC and PE which are transferred to the plasma membrane. The plasma membrane synthesis of VLCFA-containing PS could contribute to the increase of this lipid that is observed in older plant cells [24] by converting VLCFA-containing PC and VLCFA-containing PE into VLCFA-containing PS.

Acknowledgements: This work was supported by the CNRS, the Université Victor Segalen Bordeaux 2 and the Conseil Régional d'Aquitaine. P. Vincent is the recipient of a doctoral fellowship from the Ministère de la Recherche, de la Technologie et de l'Enseignement Supérieur, France. We thank C. Salat for technical assistance in preparing samples for electron microscopy, A. Descamps for photographs and J. Pope for reading the English text.

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