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Diacylglycerol pyrophosphate, a novel phospholipid compound

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In studies on lipid kinase activities of microsomal membranes from cultured plant cells a new, hitherto unknown, lipid kinase product was detected. The new phospholipid, labeled by [γ-^{32}P]ATP, could be separated from known phospholipid species by thin layer chromatography using different solvent mixtures. After partial purification of the related enzyme activity, the substrate of the unknown lipid kinase was elucidated as phosphatidic acid. With authentic phosphatidic acid and partially purified enzyme, the lipid kinase product was prepared in mg quantities and its structure was determined by mass spectrometry and NMR analyses as diacylglycerol pyrophosphate, a hitherto unknown phospholipid. The possible physiological role of this novel phospholipid metabolite is discussed.

Phospholipid; Diacylglycerol pyrophosphate; ^{31}P-NMR spectroscopy; Lipid kinase

1. INTRODUCTION

Lipid kinases which transfer the γ-phosphate of ATP onto lipid substrates, such as PI (phosphatidylinositol) kinase, PIP (phosphatidylinositol phosphate) kinase and DG (diacylglycerol) kinase, are key enzymes in phosphoinositide signal transduction [1–3]. Investigations on lipid kinase activities of suspension cultured plant cells using [γ-^{32}P]ATP and either whole cells, which were made permeable by one or two cycles of freezing and thawing [4,5], or microsomal membranes [3,6], revealed a simple pattern of labeled phospholipids separated by TLC (thin layer chromatography). Strongly labeled bands of PA (phosphatidic acid) (DG-kinase) and PIP (PI-kinase) were usually found and a weaker band of PIP_2 (PIP-kinase). Interestingly, a further strongly labeled band was obtained which had been assigned to PI up to now due to its R_F-value [4]; with two tobacco cell lines, this phospholipid was found to be the main labeled fraction [4]. In their studies on phosphoinositide kinases in plasma membranes, Memon and Boss [7] also published a labeled spot with a similar R_F-value, which they, however, assigned to lyso-PA or PA-pyrophosphate [8].

Labeling of PI with exogenous ATP, our previous assumption, is difficult to explain; the only conceivable route, i.e. de novo synthesis of PI from labeled PA [9], which again is obtained from DG and DG-kinase, is very unlikely to occur when isolated membranes are used. The findings that CTP, which is required to form CMP-PA, the substrate of PI-synthase, did not increase the intensity of the labeled band [4] was also an argument against the synthetic route. In the present work it is shown that the labeled lipid kinase product was not PI but an hitherto unknown phospholipid, diacylglycerol pyrophosphate (DGPP); this was ascertained by its properties in TLC separation, mass spectrometry and ^{31}P-NMR analysis, and by the fact that the new lipid kinase used only PA as substrate. The purification and characterization of this lipid kinase, PA-kinase, will be published in a separate work (J.B. Wissing, A. Funke and H. Behrbohm, in preparation).

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2. MATERIALS AND METHODS

2.1. *Lipid phosphorylation by [γ-^{32}P]ATP*

Origin and growth of suspension cultured *Catharanthus roseus* cells has been described [4] as well as the preparation of microsomal membrane fractions [3]. Aliquots of the latter (ca. 50 μg protein) were suspended in 200 μl 40 mM Bis-Tris (pH 6.2), 10 mM $MgCl_2$, 10 mM NaF, 0.1 mM EDTA, 1 mM spermine. 0.5 mM dithiothreitol and incubated at 30°C for 5 min. The reaction was started by addition of ATP (1 mM, with ca. 1 μCi labeled ATP) and stopped after 5 min by adding 760 μl chloroform/methanol (1:2) containing 1% concentrated HCl.

2.2. *Separation of the lipid kinase products by thin layer chromatography*

The phospholipids were extracted as described [4] and analysed by different TLC systems using 20 ×20 cm silica plates (DC-Fertigplatten, Kieselgel 60, Nr. 11845 from Merck Darmstadt) and solvent mixture I proposed by Shaikh and Palmer [10], chloroform/acetone/methanol/glacial acetic acid/water (40:15:13:12:8), mixture II from Lendrath et al. [11], chloroform/methanol/0.2 M sodium acetate pH 4 (65:25:4.3), and mixture III modified according to Hauser and Eichberg [12], chloroform/pyridine/formic acid (35:30:7). In the case of solvent mixture I, the plates were activated by a potassium oxalate solution and

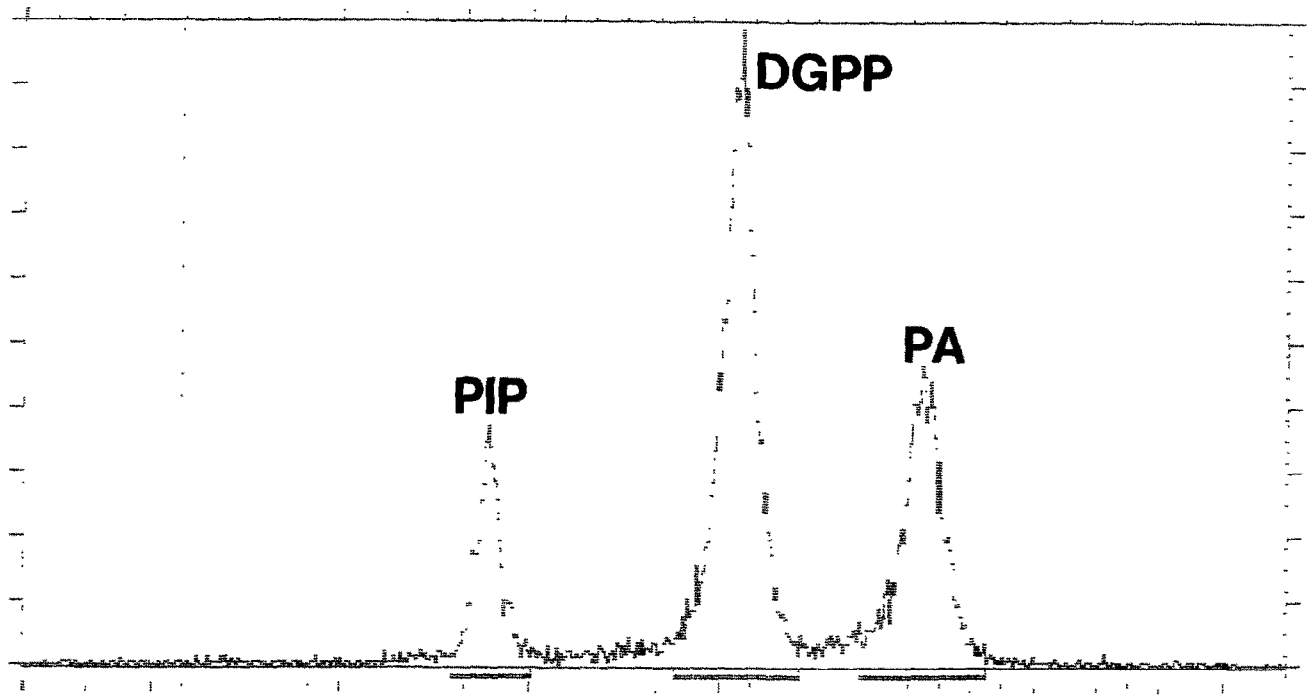


Fig. 1. Labeled products of lipid kinases present in microsomal membranes from *Catharanthus roseus* cells. The membranes were incubated with [γ-^{32}P]ATP as described in Section 2.1, without addition of exogenous lipids and the lipids separated by TLC and scanned (Section 2.2). PA is the product of DG-kinase, PIP that of PI-kinase and DGPP the product of the initially unknown lipid kinase (PA-kinase).

heating [4], whereas with mixtures II and III the plates were only heated (110°C for 20 min). The plates were scanned with a Berthold LB 512 thin layer analyzer and the spots were visualized by autoradiography (Curix RP1 X-ray films from Agfa-Gevaert). Unlabeled lipids were stained by exposing to iodine vapor.

2.3. *Determination of the lipid substrate of the unknown lipid kinase*

Crude soybean PI (50 mg) from Sigma Chemie was separated by preparative TLC (PSC-Fertigplatten, Nr. 13792 from Merck Darmstadt) using solvent mixture I or III. After staining with iodine, the bands were scraped out, the silica gel (ca. 1 ml) put onto a glass filter, the lipids extracted with chloroform (7 ml) followed by chloroform/methanol (1:2) containing 1% concentrated HCl (20 ml). After addition of water (3.5 ml) and 2 M KCl (7 ml), the phases were separated and the water phase washed twice with chloroform; the combined chloroform phases were evaporated, water was removed by lyophilisation and the lipid residue dissolved in little chloroform.

To determine the band which contained the lipid substrate, the samples were assayed with PA-kinase (see Section 2.4) and analysed by TLC as described in Sections 2.1 and 2.2. Furthermore, the active lipid fraction was deacylated [13] and the fatty acid monomethylamides were treated with methanol/HCl according to Christie [14] to form the methyl esters which were dissolved in n-hexane and analysed by GC-MS spectrometry (Carlo Erba/Kratos) using a 30 m DB1 column (J&W) for GC. The heating program started at 70°C (3 min) and the temperature was raised to 320°C (10°C/min). Mass spectrometry was performed using electron impact ionization (MS-50 FS from Kratos).

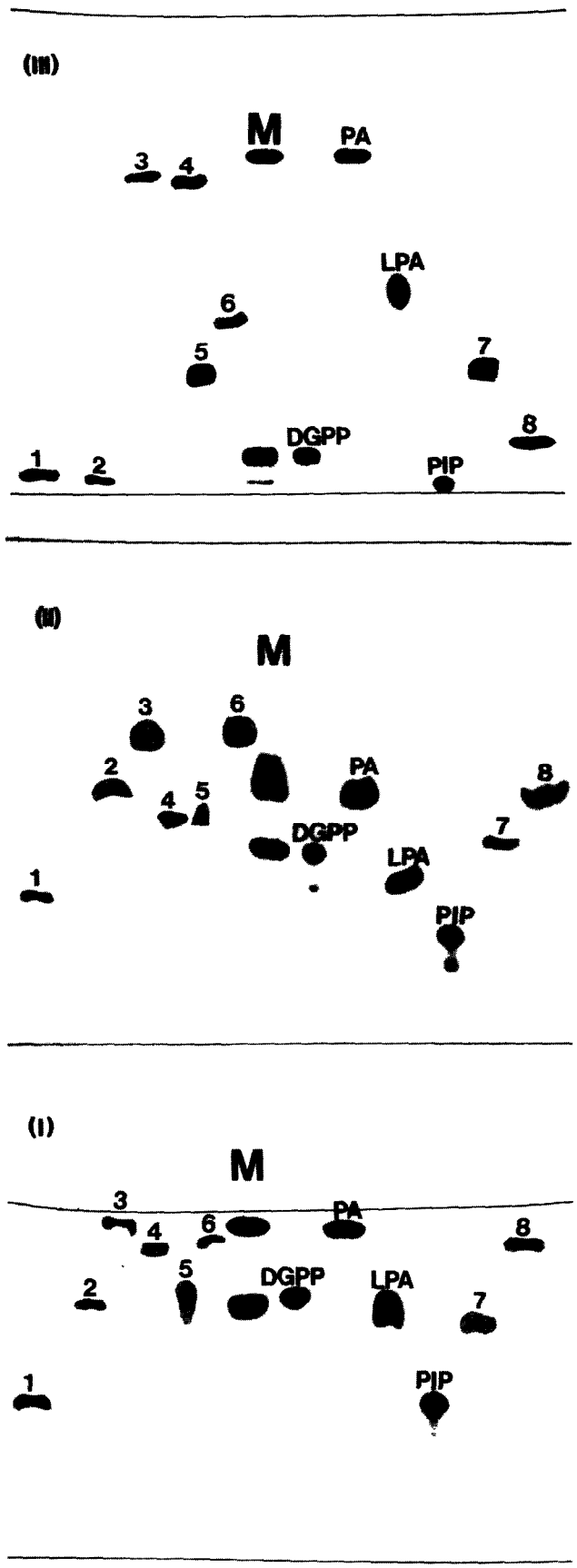
2.4. *Enzymatic preparation of DGPP from dioleoyl-PA*

25 mg dioleoyl-PA (36 μmol) were suspended in 6 ml water containing 0.05% Triton X-100, sonified and added to 30 ml buffer (20 mM imidazole of pH 6.2, 5 mM $MgCl_2$, 10 mM NaF, 0.5 mM dithiothreithol, 1 mM spermine, 0.1 mM EDTA); after addition of 12 ml water, 9 ml GTP (3 mM) and a partially purified enzyme preparation (3 ml), obtained from the microsomal fraction (J.B. Wissing, A. Funke and H. Behrbohm, to be published), the mixture was stirred in a water bath at 30°C. An aliquot (1 ml) was incubated separately after addition of a small amount of [γ-^{32}P]GTP to follow the course of the reaction. After 5 h the reaction was stopped by adding 180 ml chloroform/methanol (1:2) containing 1% concentrated HCl and 3.6 ml 100 mM EDTA; the phases were mixed, separated and the water phase washed twice with chloroform; the volume of the combined chloroform phases was reduced to 30 ml by evaporation. After adding 30 ml methanol/10 mM KH_2PO_4/chloroform (48:57:3) containing 0.25% concentrated HCl, the mixture was centrifuged for 10 min at 5,000 rpm (Heraeus-Christ minifuge 2) and the chloroform phase was evaporated. The whole procedure was performed twice transforming 50 mg dioleoyl-PA with a total enzyme activity of 0.3 μmol·min^{-1}.

The oily residue was dissolved in $CDCl_3$; ca. 8 mg were used for ^{31}P-NMR spectroscopy using NMR-tubes which were cleansed with a EDTA solution to remove paramagnetic ions. For ^{1}H- and ^{13}C-spectroscopy the surfactant (Triton X-100) was removed; an aliquot of the lipid (ca. 10 mg), dissolved in $CDCl_3$, was evaporated under nitrogen and 600 μl acetone was added. After mixing and incubation for 24 h at −20°C, the suspension was centrifuged at 0°C and the pellet dried under a stream of nitrogen, to remove the rest of acetone, and dissolved in $CDCl_3$.

2.5. *Spectroscopic methods*

Mass spectrometry of DPGG was performed by FAB-MS with negative ionization using triethylamine as matrix (Finnigan MS-spectrometer MAT 8430). Identification of PA as substrate of the PA-kinase was performed by FAB-MS with glycerol as matrix (MS-50 FS from Kratos). The NMR spectra were obtained using the Bruker spectrometers AC-200, AM-300 and WM-400. With ^{1}H-NMR and ^{13}C-NMR spectroscopy TMS (trimethylsilane) and $CDCl_3$, respectively, were used as internal references, and for ^{31}P-NMR spectroscopy 85% H_3PO_4 as external reference.



3. RESULTS AND DISCUSSION

When the lipid kinases, present in a microsomal membrane preparation, were assayed using exogenous (labeled) ATP and endogenous lipid substrates, three main labeled lipid products were obtained as shown in Fig. 1. Two of them were products of known lipid kinases such as DG-kinase (PA) and PI-kinase (PIP), whereas the product of PIP-kinase (PIP_2) was usually only scantly indicated below the PIP peak, which is typical for plant tissue [2]. There was, however, a third main peak which, due to its R_F-value, was hitherto assumed to be PI [4].

In order to decide whether the third labeled substance was indeed PI or was different from it, its chromatographic behavior was more thoroughly analysed using three TLC systems (Fig. 2). With the solvent mixture I, mainly used in our lab, authentic PI, lyso-PA and also CMP-PA overlapped with the unknown lipid, whereas PIP (and also PIP_2) and PA were clearly separated. With the solvent mixture II, lyso-PA could be separated, but only the pyridine containing mixture (III) clearly discriminated between PI, CMP-PA and the unknown lipid. It was also verified with these three TCL systems that each of the further known phospholipids was not identical with the labeled unknown lipid. These results clearly showed that the third band in Fig. 1 was not PI but an hitherto unknown phospholipid.

At this stage we were confronted with three unknown entities: the lipid kinase, its product and its substrate. With the methods described in Sections 2.1 and 2.2, it was possible to develop a quantitative enzyme assay, based on the amount of phosphate incorporated from ATP into the lipid product (Fig. 1), which allowed the partial purification of the enzyme (to be described elsewhere). However, after extraction of the enzyme from the microsomal membranes, exogenous lipid substrate, although unknown, was required. A crude lipid extract obtained from the cultured *Catharanthus roseus* cells was used first, but later on we found that crude soybean PI (Sigma) was more suitable. This lipid contained, according to the supplier's catalogue, about 50% PI and

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Fig. 2. Thin layer chromatography of the unknown lipid kinase product and reference compounds separated by three different solvent mixtures (I, II and III). The lipid kinase products were obtained from microsomal membranes of *C roseus* (lane M), separated and visualized by autoradiography as decribed in Sections 2.1 and 2.2; the low intensity of the PIP band is explained by the low pH (6.2) applied. Each of the labeled reference substances was prepared with its respective lipid kinase and [γ-^{32}P]ATP, DGPP was prepared as described in Section 2.4, labeled PA and lyso-PA (LPA) were prepared with *E. coli* diacylglycerol kinase using diacylglycerol and monoacylglycerol, respectively, as substrates [20], and PIP was prepared from PI with PI-kinase from *C roseus* [21]. The other phospholipids were separated and stained with iodine and their spots were mounted according to their R_F-values: 1, lyso-PI; 2, CMP-PA; 3, cardiolipin; 4, PG; 5, PS; 6, PE; 7, PI; 8, PC.

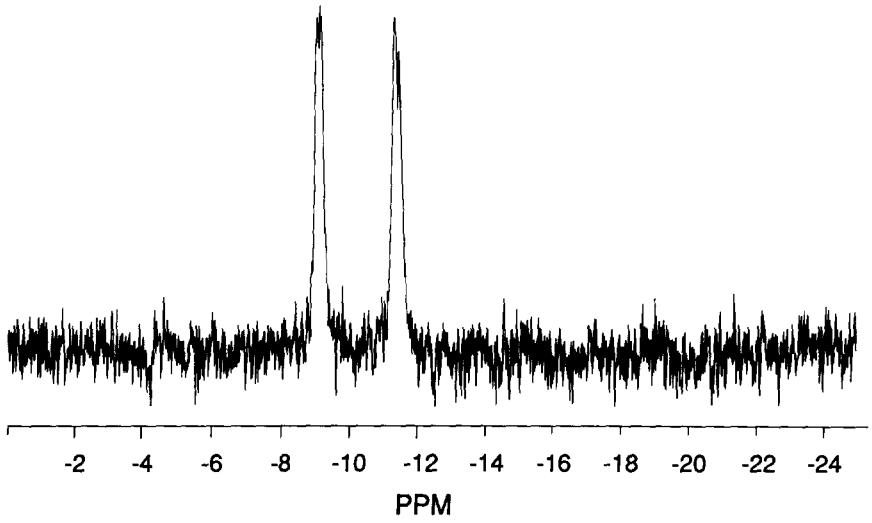

Fig. 3. ^{31}P-NMR spectrum of dioleoylglycerol pyrophosphate measured at 81 MHz and proton decoupled.

also PA and PE. We decided to use this material in the search for the unknown lipid substrate.

50 mg of soybean PI were fractionated by preparative TLC (cf. Section 2.3) and the bands were tested as substrate of the unknown lipid kinase. One active band was obtained (R_F-value 0.73 in solvent mixture I) and analyzed further by analytical TLC. When tested with all three TLC solvent mixtures (Fig. 2), this substance was identical to PA. Its identity with PA was further confirmed by specific lipid stains suitable for TLC, by ^{1}H-, ^{13}C- and ^{31}P-NMR spectrometry (data not shown). Furthermore, authentic PA proved to be the only substrate for the partially purified enzyme fraction (J.B. Wissing, to be published elsewhere). Analysis of the fatty acids, obtained by deacylation of PA purified from crude soybean PI (cf. Section 2.3), by GC-MS revealed mainly palmitic, linoleic and stearic acid.

Knowing that PA is the lipid substrate of the unknown lipid kinase (PA-kinase), authentic PA could be transformed to the unknown enzyme product. Using an enzyme fraction partially purified from microsomal membranes (total enzyme activity ca. 0.3 μmol·min^{-1}), 50 mg dioleoly-PA were quantitatively transformed to the lipid product in about 5 h (see Section 2.4). Thus enough enzyme product was obtained for its identification by spectroscopic methods.

As PA contains no other functional groups to be phosphorylated than its phosphoryl group, the product of PA-kinase was expected to be diacylglycerol pyrophosphate (DGPP), i.e. the kinase was expected to

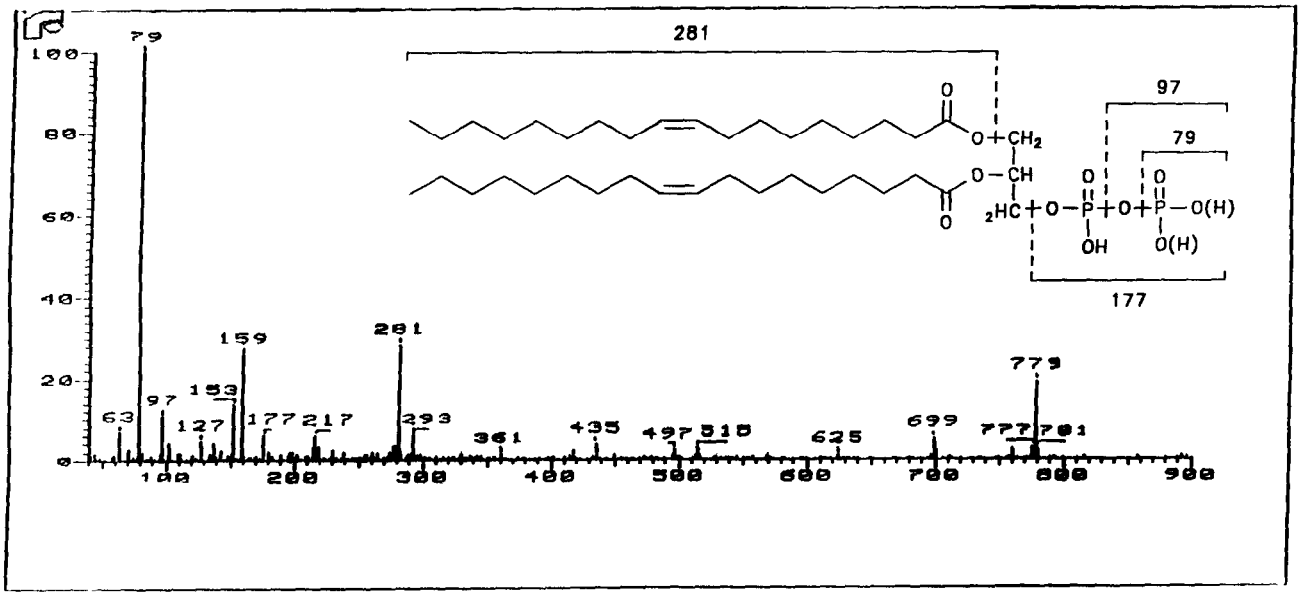

Fig. 4. FAB mass spectrum of dioleoylglycerol pyrophosphate.

transfer, in a similar manner as adenylate kinase, a phosphoryl group onto the phosphate of PA. Thus starting with dioleoyl-PA one should obtain dioleoylglycerol pyrophosphate. This was confirmed by the ^{31}P-NMR spectrum (Fig. 3) which showed two peaks with identical intensities at about −9 and −11.5 ppm which is typical for a pyrophosphate group [15]. Dioleoyl-PA showed a peak at about +1.4 ppm (not shown), typical for a phosphomonoester group, which confirmed data from other laboratories [16]. By analogy with AMP and ADP [15,17,18], the transformation of a monophosphate into a pyrophosphate should result in the formation of two peaks at higher fields as demonstrated in Fig. 3. As the αP is more strongly shielded than the βP, due to the neighboring CH_2 group, the peak at higher field (−11.5 ppm) should correspond to the αP of DGPP and the peak at lower field (−9 ppm) to the βP. This assignment is in accord with those of ADP (αP between −10 and −11 ppm and βP between −6 and −10 ppm), whereas the chemical shift of the PA phosphorous (+1.4 ppm) would correspond to that of AMP (between +1 and +4 ppm) [15,17,18]. These shifts, however, strongly depend on the pH and in the case of the pyrophosphates on the presence of divalent cations [19]. The second evidence of the formation of a pyrophosphate is its mode of coupling. Whereas AMP shows a singlet spectrum, the two peaks in ADP are doublets due to phosphorous–phosphorous coupling [15,17]. This is also evident in Fig. 3 which shows splitting of the peaks although the resolution is low; the spectrum of PA, however, was a single peak without any indication of splitting (not shown). The ^{13}C- and ^{1}H-NMR spectra were also in accord with the structure of dioleoylglycerol pyrophosphate (data not shown).

The mass spectrum of the dioleoyl derivative of DGPP, shown in Fig. 4, shows the molecule peak of DGPP $[M-H]^-$ at *m/z* 779. The fragment peak at 699 corresponds to PA, those at 515 to lyso-DGPP, 497 to dehydro-lyso-DGPP, 435 to lyso-PA, 281 to oleic acid, 177 to pyrophosphate, 153 to dehydroglycerol phosphate, 97 to $H_2PO_4^-$, 79 to the phosphoryl radical. These data further confirmed the formation of the pyrophosphate of dioleoylglycerol.

Although the structure of the novel lipid kinase product has been elucidated as diacylglycerol pyrophosphate, we do not know at present its physiological function and can only speculate. By analogy of isopentenyl pyrophosphate or phosphoribosyl pyrophosphate, DGPP might be an activated diacylglycerol and the pyrophosphate group a suitable leaving group in reactions to transfer the diacylglyceryl moiety onto suitable substrates such as e.g. cysteine side chains of a protein. Thus DGPP would be the substrate of enzymes which transfer a hydrophobic moiety to anchoring proteins within membranes. On the other hand, as DGPP is highly negatively charged on its head, one could also speculate on its role to enhance the electrical polarity of membranes.

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