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Quantitative Analysis of Changes in the Molecular Species of Glycerolipids in Cultured Cells during Signal Transduction

Chunghee Lee[1] and Amiya K. Hajra

Neuroscience Laboratory, Mental Health Research Institute, and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48104-1687

A method for the quantitative analysis of the molecular species of glycerolipids present in biological samples has been described. 1,2-Diacyl-*sn*-glycerol, either isolated from biological samples or enzymatically generated from phosphoglycerides, is benzoylated at the *sn*-3 position and then subjected to reverse-phase (C_{18}-silica) HPLC to separate the molecular species of different hydrophobicities. An internal standard (1,2-distearoyl-*sn*-glycerol) is used to identify and quantify the various species eluted from the reverse-phase column. Examples are given for the quantitative analysis of molecular species and precursor–product relationships of glycerolipids generated in SK-N-SH neuroblastoma cells after stimulation of the cell-surface muscarinic acetylcholine receptors. © 1993 Academic Press, Inc.

Diacylglycerol (DG) and phosphatidic acid (PtdOH) have multiple roles in cells. These two lipids have long been known to be the key intermediates for the biosynthesis of glycerolipids (1, 2) and in recent years have been shown to play important roles in the signal transduction process in many eukaryotic cells (3, 4). Apparently, these lipids are segregated into different cellular pools for carrying out different functions in the same cells. The DG formed after receptor activation (signal DG) has been shown to activate most isoforms of protein kinase C (5), and in some systems it is further hydrolyzed to form arachidonic acid (6), the precursor of all eicosanoids. DG is also phosphorylated to PtdOH in a reaction catalyzed by DG kinase (7). PtdOH is also directly formed from other lipids after receptor activation (see below) (8). Although no precise role of PtdOH in the signal transduction process has yet been established, PtdOH has been postulated to be a second messenger (9) and recently has been shown to activate some cytosolic protein kinases (10). Signal DG was originally believed to be formed in the plasma membrane via the receptor-mediated activation of a specific PLC (PI-PLC) that catalyzes the hydrolysis of $PtdInsP_2$ (3). However, results obtained in different laboratories have indicated that after receptor activation, other phosphoglycerides (mainly PtdCho) in addition to inositides are hydrolyzed to form DG (8, 11–14). In fact, much more of the mass of DG formed comes from PtdCho than from $PtdInsP_2$ (11–14). This receptor-mediated hydrolysis of PtdCho to DG is catalyzed either directly by PLC or PLD to PtdOH, which is further hydrolyzed to DG by phosphatidate phosphohydrolase (8, 11). Neither the site of formation nor the exact function of DG derived from PtdCho is known.

The formation of these signal lipids from different sources in different subcellular compartments and their interconversions make it difficult to study their formation and metabolism after activation of cell-surface receptors. One way to differentiate between these different precursors and products is to analyze the molecular species of these signal lipids and their putative precursors. The molecular species composition of each cellular glycerolipid is unique and remains constant during the time period of such studies. Therefore, by analyzing the molecular species profile of these lipids it is possible to deduce the precursor–product relationship between different glycerolipids. This approach has been used by several laboratories, including ours, to study the receptor-mediated formation of signal glycerides in different systems (15–19). We have developed a quantitative method for the analysis of molecular species of glycerolipids by combining and modifying methods originated in other laboratories (20). This method has been used successfully to study the formation of DG and PtdOH during activation of muscarinic acetylcholine receptors (mAChRs) of SK-N-SH neuroblastoma cells (18). Reverse-phase HPLC was employed for molecular species analysis in which separation was achieved due to differences in hydrophobicity of the various molecular species (i.e., their fatty acyl composition).

[1] Present address: NIH-NIDDK, Building 10-8C-207, 9000 Rockville Pike, Bethesda, MD 20892.

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For detection and quantification, the DG was esterified to the UV-absorbing benzoyl group and the HPLC effluent was spectrophotometrically monitored at 228 nm. All other phosphoglycerides were enzymatically converted to DG before benzoylation and HPLC separation, as shown in Fig. 1. The details are given below.

PROTOCOL

Extraction of Lipids

Mixtures of chloroform and methanol are used to extract lipids from cell or tissue homogenates. The Folch extraction method (21) is commonly employed for the extraction of lipids from solid tissues. However, the Bligh and Dyer method (22), which is a modification of Folch's, is generally used to extract lipids from cell suspensions because this method employs a relatively smaller volume of solvents. At neutral pH, all but the most acidic lipids (such as polyphosphoinositides) are extracted by this mixture of $CHCl_3$–CH_3OH. For the extraction of PtdInsP and PtdInsP$_2$, acidic $CHCl_3$–CH_3OH is generally used (23). The following method was used to extract lipids from suspensions of SK-N-SH cells.

To 1 ml of cell suspension (4–6 mg protein), 3 ml of $CHCl_3$:CH_3OH (1:2) was added, mixed, and sonicated in an ultrasonic bath (Laboratory Supply Co., Hicksville, NY) three times for 20 s each. Internal standard lipid (e.g., 1,2-distearoyl-*rac*-glycerol or 1,2-distearoyl-*sn*-glycerol-3-P) was added at this stage. The mixture was centrifuged at low speed (1000*g*, 5 min) and the supernatant was saved. To the residue, 2 ml of $CHCl_3$:CH_3OH (1:1, v/v) was added, sonicated as above, and recentrifuged. The second supernatant was combined with the first, and 1 ml of $CHCl_3$ and 1.4 ml of 0.9% aqueous NaCl were added, mixed well, and centrifuged (1000*g*, 5 min). The upper layer was removed, the lower layer was transferred to another tube, and the solvent was removed by blowing a stream of N_2 into the tubes. The residue was dissolved in a small volume of $CHCl_3$ and used immediately to isolate DG.

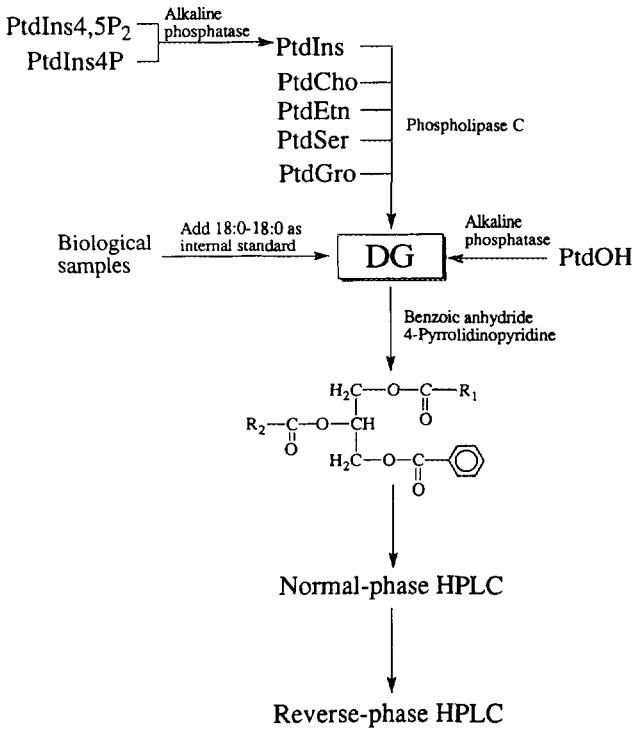

FIG. 1. Scheme for analysis of the molecular species of different glycerolipids.

The polyphosphoinositides were extracted from the solvent-extracted residue using acidified $CHCl_3$–CH_3OH. However, it should be noted that a small portion (5–10%) of these polar lipids is also extracted by the neutral $CHCl_3$–CH_3OH. The residue was dispersed in 0.1 ml of water by sonication and then 3 ml of $CHCl_3$:CH_3OH:12N HCl (200:100:1, by vol) was added, mixed, sonicated, and centrifuged at low speed. The supernatant was saved and the residue extracted again with $CHCl_3$:CH_3OH as described above. To the combined supernatants, 1 ml of $CHCl_3$ and 1.4 ml of 1 N HCl were added, mixed well, and centrifuged. The upper layer was removed and the lower layer was evaporated to dryness as described above. The residue was dissolved in a small volume of $CHCl_3$ and the inositides were purified by thin-layer chromatography as described below.

Purification of DG

The lipid extract was spotted as a band on a 20 × 20-cm thin-layer chromatography plate (E. Merck silica gel 60, 0.25 mm) at approximately 5–10 mg total lipid per plate. The plate was developed with toluene:ether:methanol (80:10:10, by vol) up to 10 cm from the origin. The DG band (R_f = 0.6), which ran above the large cholesterol band (R_f = 0.5), was localized by spraying with 0.001% Primuline in acetone–water (24) and viewing under UV light. It was identified by co-localization with authentic DG run concurrently. The DG band was scraped off and the lipid was extracted from the TLC scrapings three times with 2 ml of diethyl ether and sonication for 30 s followed by low-speed centrifugation (1000*g*, 5 min). The ether from the combined extracts was removed under N_2 and the DG was immediately used for benzoylation (see below).

Separation and Purification of Phosphoglycerides

The lipid extract was spotted as a band as described above and the plate was developed twice using the solvent system of $CHCl_3$:CH_3OH:0.25% aq KCl:ethyl acetate:isopropanol (30:9:6:18:25, by vol) to a height of 16 cm from the origin. The plate was air-dried between the two runs (25). The lipids were localized under UV light after spray-

ing with Primuline. The R_f values of PtdCho, PtdSer, PtdIns, PtdOH, and PtdEtn are 0.1, 0.15, 0.2, 0.35, and 0.45, respectively. The bands were scraped off and the lipids were isolated by extracting the silica powders three times with 2 ml of $CHCl_3$:CH_3OH (2:1) as described above. An acid extraction method using $CHCl_3$:CH_3OH:concd HCl (200:100:0.25, by vol) was employed to extract PtdIns. The HCl was removed from the extracts by adding 0.25 vol of water and discarding the upper phase. The lower $CHCl_3$ layer was dried under N_2.

Polyphosphoinositides were separated from other lipids by thin-layer chromatography on oxalate-impregnated plates (23). The lipids, obtained from the $CHCl_3$–CH_3OH–HCl extractions as described above, were spotted and the plate was developed first with $CHCl_3$:CH_3OH:7.4 M aq NH_4OH:H_2O (40:48:5:10, by vol), air-dried, and developed again with *n*-propanol:7.4 M NH_4OH:water (65:20:15, by vol) (26). Lipid bands were localized after spraying with Primuline, and PtdInsP and $PtdInsP_2$ were extracted from TLC scrapings by dispersing them in 3 ml of $CHCl_3$:CH_3OH:12 N HCl (66:33:0.25, by vol), followed by centrifugation. The supernatant was saved and the residue extracted again with acidic $CHCl_3$–CH_3OH. To the combined extracts 0.25 vol of water was added, mixed well, and centrifuged and the upper layer was removed. The lower layer was filtered through a 0.45-μm polytetrafluoroethylene (PTFE) filter and the filtrate was then dried under N_2.

Hydrolysis of Phosphomonoesters by Alkaline Phosphatase

The polyphosphoinositides are not hydrolyzed by bacterial phospholipase C; therefore, these lipids were first enzymatically dephosphorylated to PtdIns, which was then further hydrolyzed by PLC.[2] PtdOH was also enzymatically hydrolyzed to DG. The lipids were emulsified in sodium deoxycholate and hydrolyzed by alkaline phosphatase (27) as described below.

The lipids were dried under N_2 in Reacti-Vials (Pierce Chemical Co.), 0.5 ml of a mixture containing 10 mM Tris–HCl buffer (pH 7.4), sodium deoxycholate (3 mM), and alkaline phosphatase (10 U, calf intestinal, Sigma) was added, and the mixture was magnetically stirred at 37°C for 2 h. The reaction was stopped by adding 5.0 ml $CHCl_3$:CH_3OH (2:1) followed by 1 ml of 1 N HCl. After mixing and centrifuging, the upper layer was removed and the lower layer was transferred to another vial. The $CHCl_3$ was removed under N_2. PtdIns formed from PtdInsP and $PtdInsP_2$ was purified by TLC as described above. The DG formed from PtdOH was also purified by TLC and then benzoylated.

Hydrolysis of Phosphoglycerides to DG

Purified PtdCho, PtdIns, PtdSer, PtdGro, and PtdEtn were emulsified by sonication in 0.5 ml of a solution containing Tris–HCl and sodium deoxycholate as described above. These mixed micelles were then incubated with 10 U of phospholipase C from *Bacillus cereus* (Sigma) at 37°C for 2 h (28). The reaction was terminated by the addition 2.4 ml $CHCl_3$:CH_3OH (1:1) followed by 0.5 ml of 1 N HCl. After mixing and centrifugation, DG from the lower layer was purified by TLC using toluene:ether:methanol (80:10:10, by vol) as the mobile phase as described above.

Benzoylation of DG

The purified DG in ether was transferred to 1.6 × 18-cm screw-topped tubes and the solvent was removed by blowing a stream of dry N_2 into the tubes. To the dry thin film of DG, 100 μl of dry benzene (distilled from CaH_2) and 20 μl of a solution containing 7 μmol of 4-pyrrolidinopyridine (Sigma) and 5.5 μmol of benzoic anhydride (Sigma) in dry benzene were added. The components were mixed by vortexing, dry N_2 was blown in the tubes to displace the air, and the tubes were capped with Teflon-lined screw caps. The mixtures were incubated at room temperature for 1 h with mixing after every 10–15 min. The reaction was stopped by adding 0.1 ml of methanol, and the reaction mixture was spotted as a band on a thin-layer plate (E. Merck silica gel 60), which was then developed with hexane:diethyl ether:7.4 M NH_4OH (55:45:1). The benzoyl DG band (R_f = 0.65) was scraped off, extracted three times with 2 ml of diethyl ether, and further purified by normal-phase HPLC.

Purification of Benzoyl DG by Normal-Phase HPLC[3]

The normal-phase HPLC was performed using a silica gel column (25 × 0.46 cm, 5-μm-diameter particles, Rainin) attached to a solvent delivery system (Waters) and to a recording spectrophotometer (Kratos Spectraflow) for monitoring the effluent. The benzoyl DG in ether was filtered through a 0.45-μm PTFE filter to remove suspended particles, and the filtrate was dried under N_2. The residue was dissolved in cyclohexane and an aliquot (20 μl) was injected into the column. Benzoyl DG was eluted isocratically using 3.7% diethyl ether in cyclohexane at a flow rate of 1 ml/min at room temperature. The elution was monitored at 228 nm and the benzoyl DG peak (R_t = 13 min) was collected and dried under N_2. This step removes large amounts of UV-absorbing material and also if present, the alkyl (R_t = 6.7 min) and 1-alk-1′-enyl (R_t = 5.6 min) analogs of benzoylated DG.

[2] The two steps for the enzymatic conversion of the phosphoinositides to DG can be combined as follows: The dry lipids are emulsified in 0.5 ml of a mixture containing 10 mM Tris–HCl buffer (pH 7.4) and sodium deoxycholate (3 mM) by sonicating in a bath sonicator. Alkaline phosphatase (10 U, calf intestine, Sigma) and phospholipase C (10 U, *B. cereus*, Sigma) are added and the mixtures magnetically stirred at 37°C for 2 h. The DG is extracted from the reaction mixture as described in the text.

[3] This step can be omitted if the noise at the origin of the reverse-phase HPLC is not objectionable.

Separation of Molecular Species of Benzoyl DG by Reverse-Phase HPLC

The benzoyl DG fraction collected from normal phase HPLC was dried, dissolved in a small volume of acetonitrile, and injected into an octadecyl silica gel column (25 × 0.46 cm, 5-μm-diameter particles, Beckman Instruments). The species were eluted isocratically using acetonitrile:isopropanol (70:30) with a flow rate of 1 ml/min at room temperature. The eluate was monitored at 228 nm and the peak areas were integrated using an electronic integrator (Nelson Analytical) attached to the monitoring system. Typical results obtained for the DG and PtdOH of SK-N-SH cells are shown in Fig. 2.

Characterization and Identification of the Peaks after Reverse-Phase HPLC

The peaks were tentatively identified by comparing the retention times of the unknowns with that of standard DG of known composition. Since separation in this system is based on the hydrophobic interaction of the molecules with the stationary phase, the retention time will increase with increasing chain length and decrease with the presence of double bonds in the chain. The positions of the fatty acids in the glycerol moiety and the position of the double bond in the alkyl chain also determine the overall hydrophobicity of the molecules. Patton *et al.* (29) described a useful graphical method of identifying different molecular species by plotting the carbon number of the acyl chain at C-1 of the glycerol vs the logarithm of retention time. This yields a series of parallel lines of isohydrophobicity depending on the carbon number and the degree of unsaturation of the acyl group present at the C-2 of the glycerol moiety.

For accurate identification, the fatty acid composition of the benzoyl DGs present in each peak should be analyzed by gas–liquid chromatography. Each peak collected from the HPLC column was dried and methanolyzed by heating for 15 min at 60°C with 0.5 ml of 13% BF_3 in methanol (Sigma). After the reaction was complete, 2 ml of hexane and 1.5 ml of water were added to the mixture, and tubes were mixed well and centrifuged. The upper hexane layer was transferred to another tube and the lower layer was extracted twice more with hexane. The combined hexane layers were dried under N_2, dissolved in a small volume of dichloromethane, and injected into the gas chromatography column. Gas chromatography was performed by using a Hewlett–Packard Model 5800 apparatus, equipped with a fused silica capillary column (30 m × 0.2 mm) coated with cyanopropyl silicone (SP 2330, Supelco Co.), and a flame ionization detector. The peaks were identified by comparing the retention time values to those of known standards. When a mixture of molecular species is present in one HPLC peak, the quantitative composition of each one can be deduced from the number and relative amounts of each fatty acid present. A 1:1 molar ratio of any two particular fatty acids indicates the composition of a particular molecular species. In addition to the commonly known fatty acids, the lipids of SK-N-SH cells, especially the inositides, contained a number of unusual fatty acids, such as 20:3, *n*-9 (Mead acid). Apparently, for these fast-growing tumor cells, these unusual *n*-9 polyunsaturated fatty acids were synthesized by the growing cells due to an insufficiency of essential fatty acids in the medium.

Quantification of Molecular Species

The amount of DG present under an individual HPLC peak can be calculated by comparing the area under the

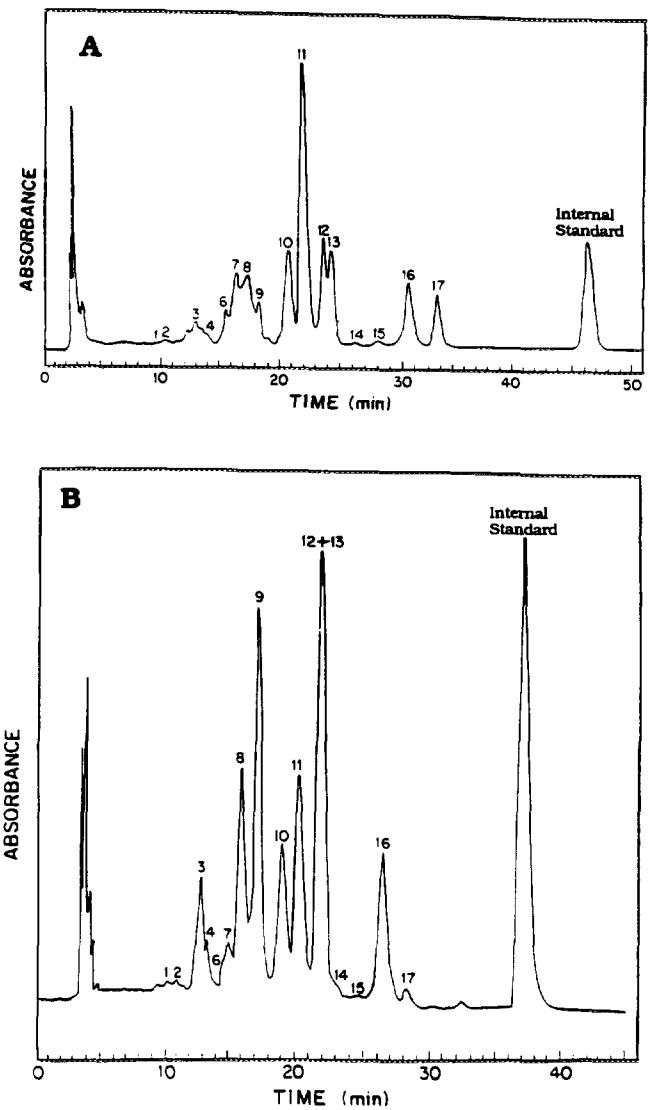

FIG. 2. Separation of molecular species of 1,2-diacyl-3-benzoyl-*sn*-glycerol by reverse-phase HPLC according to their hydrophobicity. The samples are derived from either DG (A) or PtdOH (B) from SK-N-SH neuroblastoma cells after stimulation with carbamoylcholine for 5 min. The peak numbers correspond to the species indicated in Table 1. Benzoylated distearoylglycerol was the internal standard.

peak with the area under the internal standard peak. Because the absorbance at 228 nm is due to the benzoyl moiety ($A_{228} = 1.3 \times 10^4\ M^{-1}\ cm^{-1}$), the calculation should be done on a molar basis. As the amount of internal standard added during lipid extraction is known, the molar amount of DG present in the unknown peak can be calculated by comparing the areas under each peak. Results obtained from the analysis of molecular species of PtdCho and PtdIns of the SK-N-SH cells are given in Table 1.

Data Analysis

Because both the molecular species composition of the precursor lipids (PtdCho and PtdIns) and the quantitative changes in the molecular species of the signal lipids (DG, PtdOH) are known, we attempted a simple calculation to determine the relative contribution of each of the precursor lipids to the formation of the DG and PtdOH at different time points after stimulation with the agonists. For these calculations, it is assumed that after receptor activation, the DG species originated only from PtdCho and PtdIns (and other inositides) and that the molecular species compositions of PtdCho and PtdIns are the same in all cellular compartments.

Suppose that K is the fraction of DG derived from inositides and that $1 - K$ is the fraction of DG derived from PtdCho. If a is the mol% of a particular species of increased DG, b is the mol% of the same species in PtdIns, and c is the mol% of the same species in PtdCho, then

$$a = K \times b + (1 - K) \times c.$$

Solving this equation,

$$K = (a - c)/(b - c).$$

Because a, b, and c can be experimentally determined, K can be calculated.

Using these calculations, it was found that after the stimulation of mAChRs of the SK-N-SH cells for a short time (<30 s) almost all of the DG formed came from the inositides. However, upon chronic stimulation of the receptors, a large portion of DG was calculated to be derived from the hydrolysis of PtdCho. One such calculation is shown in Table 2, where it is seen that after stimulation for 5 min, the molecular species composition of DG indicates that both PtdIns and PtdCho are precursors. With few exceptions, the calculated values show that about 30% of each molecular species is derived from PtdIns (Table 2). However, when these calculations were made for the increased amounts of different molecular species of PtdOH for the same time period (5 min), almost all (>90%) of the molecular species appear to be derived from inositides (Table 3).

TABLE 1

Molecular Species of Phosphatidylcholine and Phosphatidylinositol of SK-N-SH Cells

Peak No.	Species	PtdCho	PtdIns
1	18:1-22:6 (*n*-3)	0.88 ± 0.04	0.33 ± 0.03
2	16:0-22:6 (*n*-3)	0.52 ± 0.03	0.28 ± 0.03
3	16:1-16:1 (*n*-7)	1.31 ± 0.07	
	18:1-22:5 (*n*-3)	0.15 ± 0.01	
3′	14:0-16:1	1.79 ± 0.10	
	18:1-20:4 (*n*-6)	0.85 ± 0.03	8.56 ± 0.26
	16:0-22:5 (*n*-3)	0.27 ± 0.10	
4	16:0-20:4 (*n*-6)	0.20 ± 0.02	2.10 ± 0.10
5	16:0-	0.82 ± 0.06	Trace
6	18:1-X[a]	3.41 ± 0.11	0.37 ± 0.04
7	14:0-18:1	1.82 ± 0.05	0.84 ± 0.09
	16:1-18:1	7.16 ± 0.21	
8	14:0-16:0	1.84 ± 0.04	
	16:0-16:1	9.51 ± 0.22	2.07 ± 0.10
	18:1-20:3 (*n*-9)	0.11 ± 0.01	16.70 ± 0.10
9	18:0-20:4 (*n*-6)	0.21 ± 0.04	18.40 ± 0.16
	15:0-18:1	0.52 ± 0.09	
	16:0-20:3 (*n*-9)	0.07 ± 0.01	3.81 ± 0.02
10	18:1-18:1	12.48 ± 0.21	5.65 ± 0.06
11	16:0-18:1	35.24 ± 0.91	3.97 ± 0.04
	18:0-X		0.86 ± 0.01
12	16:0-16:0	7.46 ± 0.01	Trace
13	18:0-20:3 (*n*-9)	0.50 ± 0.01	32.82 ± 0.33
14	17:0-18:1	0.64 ± 0.05	0.23 ± 0.03
15	18:0-20:1	1.05 ± 0.10	0.40 ± 0.04
16	18:0-18:1	9.56 ± 0.46	1.50 ± 0.20
	16:0-20:1	0.74 ± 0.05	0.10 ± 0.02
17	16:0-18:0	1.43 ± 0.05	0.88 ± 0.12
18	18:0-20:1	0.20 ± 0.05	0.11 ± 0.01

Note. The purified phospholipids were hydrolyzed with phospholipase C and the resulting DGs were benzoylated and subjected to reverse-phase HPLC separation as described in the text. The fatty acid composition of each peak from HPLC eluates was analyzed by GLC. The "*n*-minus" nomenclature for the abbreviation of fatty acids (i.e., number of carbon atoms (*n*):number of double bonds and the position of the last double bond (*n*-minus) from the caboxyl end) was used. The fatty acid present at C-1 of glycerol followed by that present at C-2 is given, the positions being assigned arbitrarily. The results expressed as mol% are the means ± SE for three experiments. Peak numbering is the same as that indicated in Fig. 2.

[a] X is a mixture of 18:2, 20:3 (*n*-6), and 22:4 (*n*-6).

DISCUSSION

A number of methods for the separation of molecular species of DG have been described. Most of these methods involve reverse-phase HPLC of different DG derivatives (15, 16, 18, 19). Raben and co-workers have described a gas-chromatographic method for the separation of DG (17), which is described elsewhere in this issue [Leach and Raben (30)]. We chose the present method because only mild conditions are needed to make the derivative and because the benzoyl derivative is stable and has a high molar absorbance (13,400 $M^{-1}\ cm^{-1}$). With a sensitive optical HPLC detector, as little as 20 pmol of individual

TABLE 2

Relative Contribution of PtdIns and PtdCho to the DG Formed in SK-N-SH Cells after Stimulation with Carbachol for 5 min

Peak No.	Species	DG (nmol) 0 min	DG (nmol) 5 min	DG (nmol) 5 − 0 min	ΔDG (mol %) *a*	PtdIns (mol %) *b*	PtdCho (mol %) *c*	Calculated fraction derived from PtdIns $(a - c)/(b - c)$
6	18:1-X	0.38	0.46	0.08	3.4	0.4	4.2	0.21
8	18:1-20:3/16:0-16:1	0.40	0.69	0.29	13.1	19.6	10.5	0.29
9	18:0-20:4/16:0-20:3	0.11	0.25	0.14	6.4	26.1	1.0	0.22
10	18:1-18:1	0.83	1.08	0.25	11.7	6.6	15.5	0.43
11	18:0-X/16:0-18:1	1.15	1.83	0.68	31.0	5.6	43.6	0.33
12	16:0-16:0	0.52	0.66	0.15	6.8	0.1	10.0	0.32
13	18:0-20:3	0.17	0.43	0.26	11.7	38.6	0.6	0.29
16	18:0-18:1	0.44	0.65	0.21	9.2	1.9	12.8	0.33
17	16:0-18:0	0.28	0.43	0.15	6.8	1.1	1.7	−8.50

Note. The cells were incubated with (5 min) or without (0 min) 1 mM carbachol and the DG from the cells was analyzed as described in the text. The increased amounts of each molecular species of DG were determined by subtracting that amount in the control sample from that in the stimulated cells. The molecular species composition of the increased DG in mol% was calculated and shown in *a*. The composition of DG was then compared to the molecular species compositions of PtdIns (*b*) and PtdCho (*c*), and the fraction of each molecular species of DG obtained from each of these lipids was calculated as described in the text and given in the last column.

component can be quantified with this method. DG coupled to a fluorescent group (31) might be useful for a more sensitive detection and estimation of individual molecular species of lipids. However, the drastic conditions necessary for acylation of DG with the fluorophore (31) may nullify the sensitivity advantage gained. We found that when acid chloride is used for acylation of DG at high temperatures (19, 29, 31), a large fraction of 1,2-DG is isomerized to 1,3-DG. In reverse-phase HPLC, the benzoylated 1,3-DG derivatives run faster than the corresponding 1,2-isomers. Therefore, to achieve a good resolution of the molecular species, it is necessary to separate the 1,3-DG isomer from the 1,2-isomer before the reverse-phase HPLC step.

Isomerization of the 1,2-species to the 1,3-species does occur in the present method to a limited extent. The 1,3-DG is separated from the 1,2-DG on TLC and the respective benzoyl derivatives are separated from each other in the normal-phase HPLC. Because internal standard DG undergoes the same isomerization process, theoretically to the same extent as other DG species, this does not affect the quantitative analysis of the molecular spe-

TABLE 3

Comparison of the Molecular Species Composition of PtdIns and PtdCho with That of PtdOH Formed after Stimulation of mAChRs in SK-N-SH Cells

Peak No.	Species	0 min (pmol/mg)	5 min (pmol/mg)	Diff (5 − 0 min) (pmol/mg)	ΔPtdOH (mol%) *a*	PtdIns (mol%) *b*	PtdCho (mol%) *c*	Calculated fraction derived from PtdIns $(a - c)/(b - c)$
3	18:1-20:4	5.74	29.07	23.33	5.4	8.5	1.6	0.55
4	16:1-20:4	1.81	12.29	10.48	2.4	2.1	0.2	1.18
6 and 7	16:1-18:1	5.67	14.97	9.30	2.2	2.8	15.3	1.05
8	18:1-20:3	23.07	73.66	50.59	11.8	12.9	0.1	0.91
9	18:1-20:4	10.41	111.82	101.41	23.6	22.2	0.2	1.06
10	18:1-18:1	22.13	49.27	27.14	6.3	5.7	15.3	0.94
11	16:0-18:1	58.36	79.54	21.18	4.9	4.8	43.2	1.00
12 and 13	18:1-20:3	27.45	182.7	155.25	36.1	36.0	9.8	1.00
16	18:0-18:1	36.78	68.32	31.54	7.3	6.1	14.1	0.85

Note. The comparison was made as described in the text and in the note to Table 2. The calculated values of the fraction for each molecular species of PtdOH derived from PtdIns are shown in the last column.

cies. However, care should be taken to minimize this isomerization process to prevent sample loss and contamination with the unwanted isomer. We found that rapid processing of DG up to the benzoylation step results in very little (0–5%) conversion of DG to the 1,3-isomer. If it is necessary to store the DG sample before benzoylation, then it should be stored under acidic conditions (pH 3–5)because the rate of isomerization is enhanced at alkaline and strong acid pH. Addition of a trace of acetic acid to the DG in $CHCl_3$ solution retards the isomerization process.

The benzoyl derivatives may be stored for a short time before HPLC, provided that precautions are taken to prevent oxidation of the unsaturated fatty acid. Storing in organic solvent at low temperature under a N_2 or argon atmosphere is advised. Oxidation of polyunsaturated fatty acids during the processing described here is negligible, as has been shown by using 1-stearoyl-2-arachidonoyl-*sn*-glycerol (20).

One advantage of the HPLC method is that, after separation, the species present in each peak can be further analyzed. Compounds having the same hydrophobicity migrate together as one peak in reverse-phase HPLC. Most of the species can be identified by comparison of their retention times with those of known standards and from the hydrophobicity plot as described above. However, these identifications should be confirmed by the gas-chromatographic analysis of fatty acid composition of the DG present in each peak. Cultured cells may contain some unusual fatty acids not commonly present in biological tissues. As mentioned above, we have found fairly large amounts of *n*-9 polyunsaturated fatty acids in the inositides of cultured SK-N-SH cells (18).

The quantification of total and individual species of DG is best achieved by using an internal standard. The internal standard should be added during the extraction of the lipid so that the recovery of the standard at each step will be the same as that of the unknown species. The amount of unknowns present in the original extract can be calculated directly by comparing the areas under the peaks. The comparison should be done on a molar basis because absorbance at 228 nm is due to the benzoyl group only (1 mol/mol of DG) regardless of the fatty acids present in a particular species.

The internal standard should be chosen carefully so that on HPLC it does not overlap with the naturally occurring species present in the system under study. We have chosen distearoyl DG (18:0-18:0) or the corresponding phosphatidic acid as the internal standard because each is either absent or present in very low amounts in most biological systems. However, the absence of 18:0-18:0 should be verified by analyzing the sample first without any addition of the internal standard.[4] Several 1,2-DGs are available from commercial sources (Sigma, Avanti) and they can also be generated by PLC-catalyzed hydrolysis of synthetic PtdCho, also available commercially.

The calculation given above for determining the precursor–product relationship between different lipids works well with homogeneous populations of cultured cells in which the molecular species composition of a particular lipid is similar in different subcellular compartments. We found that this type of calculation is not applicable to complex biological samples, such as those from brain, in which many types of cells are present. However, within certain limitations, meaningful conclusions regarding the sources of DG and PtdOH and their interconversions in the cultured cells can be drawn. For example, from the data and calculations presented in Tables 2 and 3, we concluded that initially (<30 s) after receptor stimulation, inositides were preferentially hydrolyzed to DG (18). After a certain interval, PtdCho was also hydrolyzed to DG at a relatively higher rate than the inositides. However, the DG from inositides, but not the DG from PtdCho, is phosphorylated to PtdOH (18). These conclusions have been verified in our laboratory (32) and others (33) using different experimental approaches, indicating that the conclusions derived from the analysis of our data, as described above, are valid. However, it should be emphasized that because of the presence of multiple cellular pools of the same lipid, this precursor–product relationship is only approximate and such conclusions should be verified by employing other experimental means.

[4] Recently, in cultured Madine–Darby canine kidney cells, we found fairly large amounts (~5% of the total) of 18:0-18:0 DG species (C. Lee, A. K. Hajra, and J. Shayman, unpublished results).

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