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Complementary chromatographic analysis of free diacylglycerols and potential glycerophospholipid precursors in human SH-SY5Y neuroblastoma cells following incubation with lithium chloride

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Abstract

We performed detailed chromatographic analyses on the molecular species of the major glycerophospholipids (GPLs) and free *sn*-1,2-diacylglycerols (DAGs) from SH-SY5Y human neuroblastoma cells following incubation with or without LiCl. For this comparison the inositol, choline, ethanolamine and serine GPLs were dephosphorylated with phospholipase C and the released *sn*-1,2-diacylglycerols along with the DAGs were subjected to high-temperature GLC on polar and non-polar capillary columns as their trimethylsilyl and *tert.*-butyldimethylsilyl ethers. A 30-min incubation with 10 m*M* LiCl increased the total amount of human neuroblastoma DAGs by 32–58% ($P < 0.05$) to 2.6 pmol/μg cell protein. This was accompanied by a limited qualitative shift in the molecular species pattern, the most obvious of which was the increase (13%) in the major saturated–polyunsaturated molecular species and the *ca.* 46% increase in the minor 18:1–18:1 species over control levels. The DAGs originated mainly from the inositol GPLs (IGPLs), as indicated by the high levels of the characteristic 18:0–20:4n6 (18:0–20:3n9) species in both IGPLs and DAGs, and to a lesser extent from the choline GPLs (CGPLs), as indicated by the high proportion in CGPLs of the oligoenoic species, which were largely absent from IGPLs. Alkenylacylglycerols were not detected in DAGs, although they made up some 60% of the total ethanolamine GPLs (EGPLs). No significant changes in the molecular species composition of the cellular GPLs, including IGPLs, were detected after exposure to LiCl.

1. Introduction

Recently lithium-induced increases in diacylglycerol (DAG) levels have been reported [1] in mouse neuroblastoma × rat glioma hybrid cells of a NG108-15 clone and this has raised the

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possibility that the physiological effects of Li^+ ions may involve protein kinase C activation. Since a comparison of the amounts of inositol phosphate and DAGs released in response to Li^+ showed a significant discrepancy in favor of the DAGs in NG-108 cells, it was suggested [1] that sources other than inositol glycerophospholipids (IGPL) were contributing the diacylglycerols. The origin of the DAGs was not investigated. The DAG species, which activate protein kinase C were once thought to come exclusively from IGPLs via a specific phospholipase C with the simultaneous production of the second messenger, inositol 1,4,5-triphosphate [2]. More recent evidence suggests the participation of cellular choline glycerophospholipids (CGPLs) in this process [3,4], stimulated by growth hormones in some mammalian cells [5–7]. This has necessitated the development of improved methods for establishing a precursor–product relationship between the cellular GPL and DAG.

In the present study we demonstrate that comparison of the molecular species composition of the DAGs and individual GPL classes by complementary chromatographic techniques can provide estimates of both qualitative and quantitative contributions from different sources. The methodology is illustrated by examining the effect of Li^+ on the glycerolipid composition of human SH-SY5Y neuroblastoma cells. No previous analyses of the molecular species of glycerolipids from lithium treated cells have been performed.

2. Experimental

2.1. Materials

The reagents and biochemicals, including cell growth media, were as previously described [8]. LiCl and bradykinin were from Sigma (St. Louis, MO, USA). *sn*-1,2-Dioleoylglycerol was obtained from Serdary Research Labs. (London, Ont., Canada). Reference *sn*-1,2-diacylglycerols and *sn*-1-alk-1-enyl 2-acylglycerols were prepared from egg yolk CGPLs and EGPLs, respectively, by digestion with phospholipase C from *Bacillus cereus* and normal-phase HPLC [8]. Reference IGPLs and SGPLs (bovine brain) were from Sigma.

2.2. Cell culture

Human neuroblastoma SH-SY5Y cells [9] were grown to confluency in RPM I medium (7% fetal calf serum) as previously described [10], when they contained 350–400 pg protein/cell. Serum deprived cells were incubated 72 h prior to extraction with 4% and 24 h prior to extraction with 1% fetal calf serum (plus fresh 1% glutamine) in the media. Experiments were initiated by removing the culture medium and washing the cell surface gently with 2×5 ml of ice-cold KRGH buffer (150 m*M* NaCl, 1 m*M* $CaCl_2$, 5 m*M* KCl, 1.2 m*M* $MgCl_2$, 5 m*M* glucose and 25 m*M* HEPES, adjusted to pH 7.3 with dilute NaOH). A 5-ml volume of KRGH buffer was then added to each Petri dish plus or minus 10 m*M* LiCl. Cultures were further incubated for 30 min at 37°C. The KRGH buffers (plus or minus LiCl) were then aspirated and ice-cold methanol (2×2 ml) was added. The cellular material was scraped from the Petri dishes with a rubber policeman into extraction–centrifugation tubes.

2.3. Extraction of lipids

The cellular material from one 10-cm plate was suspended in 4 ml of ice-cold methanol in a 25-ml centrifuge tube and diluted with 8 ml of chloroform and 1.2 ml of 0.1 *M* HCl added along with 10 mg of BHT as antioxidant [8]. The tubes were vortex-mixed vigorously and allowed to stand at 4°C until phases were well separated. The lower organic phase was then removed and reduced to a small volume under nitrogen gas.

2.4. Separation of lipid classes

Neutral lipids were isolated from the dried total lipid extracts by silicic acid column chromatography using 10 column volumes of chloroform [8]. The phospholipids were removed with 5 column volumes of acetone and 10 column

volumes of methanol [11]. An aliquot of the total neutral lipid fraction was used for a quantitative assay of DAGs by *E. coli* kinase as described below. The remainder of free diacylglycerols were resolved into *sn*-1,2- and X-1,3-diacylglycerols by TLC on Silica Gel G plates impregnated with 5% boric acid using chloroform–acetone (96:4, v/v) containing 0.1% BHT [12]. In this system, X-1,3-diacylglycerols (R_F 0.79–0.73) and *sn*-1-alkenyl-2-acylglycerols (R_F 0.70–0.65) are well separated from *sn*-1,2-diacylglycerols (R_F 0.60–0.56). The chromatographic lanes containing samples were covered completely with aluminum foil and the lanes containing standards were sprayed with 2,7-dichlorofluorescein (Sigma). The DAGs were removed from each lane immediately after chromatography with chloroform–acetone (90:10, v/v) containing antioxidant. The slurry was centrifuged at 500 *g* for 10 min to settle the silica gel. The supernatant was transferred to 10-ml screw-cap vials for drying and preparation of the TBDMS ethers.

The GPLs were resolved into the individual classes by DEAE cellulose column chromatography essentially as described by Christie [11]. CGPLs were eluted with chloroform–CH_3OH (9:1, v/v), EGPLs with chloroform–methanol (1:1, v/v), SGPLs with glacial acetic acid, and IGPLs with chloroform–methanol (4:1, v/v) made 0.05 *M* with NH_4OAc and 0.56% with respect to NH_4OH. Residual salts were removed from the last fraction by rapid extraction and partition using 0.9% NaCl.

The GPL fractions were purified by TLC on commercially prepared Silica Gel G plates (20 × 20 cm, 250 μm thick layer) supplied by Analtech (Fisher Scientific, Boston, MA, USA). CGPLs (R_F 0.13) and EGPLs (R_F 0.59) were isolated using chloroform–methanol–water (25:10:1, v/v, plus BHT) as the solvent [13]. IGPLs (R_F 0.32) was purified similarly using chloroform–methanol–acetic acid–water (50:30:8:4, v/v, plus BHT) [14]. The phospholipids were located by spraying with 0.1% 2,7-dichlorofluorescein in 95% methanol and by comparing the R_F values to those of reference standards. The phospholipids were recovered from the silica gel scrapings by extraction with chloroform–methanol (1:1, v/v) immediately after separation and before the plate had dried.

2.5. Preparation of derivatives

Fatty acid methyl esters were prepared by transmethylation of the glycerolipids with 6% H_2SO_4 in methanol [15]. The methyl esters were extracted with hexane. *tert.*-Butyldimethylsilyl (TBDMS) ethers of diacylglycerols were prepared by reaction with a solution of *tert.*-butyldimethylchlorosilane–imidazole (1:2.5, mol/mol) in dimethyl-formamide at 80°C for 20 min [16]. The mixture was extracted with chloroform–methanol (2:1, v/v) and washed with water. Trimethylsilyl (TMS) ethers of the diacylglycerols were prepared by reaction with a solution of pyridine–bis(trimethylsilyl)–trifluoroacetamide–trimethylchlorosilane (50:49:1, v/v), which yields volatile by-products [17].

2.6. Analysis of molecular species of free DAG as TBDMS ethers

Prior to GLC resolution of the molecular species, the TBDMS ethers of the free diacylglycerols were purified by normal-phase HPLC using a Spherisorb 3 μm column (100 × 4.6 mm I.D.) and hexane–isopropanol (99.8:0.2, v/v) as eluting solvent at 0.5 ml/min [18]. The pure TBDMS ethers of DAG were collected between 6 to 9 min, corresponding to the TBDMS ether of standard *sn*-1,2-dioleoylglycerol at 8 min. The peaks were detected at 214 nm.

The purified TBDMS ethers were resolved by carbon number using a non-polar GLC column (8 m × 0.32 mm I.D. fused-silica capillary) coated with a crosslinked 5% phenylmethylsilicone (Hewlett–Packard). The sample was injected on-column and the temperature was programmed in four steps from 40 to 350°C: 40 to 150°C at 30°C/min; then to 230°C at 20°C/min; then to 280°C at 10°C/min; and to 340°C at 5°C/min. The carrier gas was hydrogen at 41.4 kPa head pressure. The purified TBDMS ethers were resolved into molecular species by GLC on

a polar capillary column (15 m × 0.32 mm I.D. coated) with SP2380 (Supelco, Oakville, Ont., Canada), operated isothermally (260°C) or temperature programmed from 240°C to 260°C at 5°C/min with hydrogen as carrier gas at 13.8 kPa head pressure [19]. The sample was introduced by split injection (split ratio 7:1).

2.7. Analysis of GPL as TMS ethers of diacylglycerols

For this purpose the purified GPLs were dephosphorylated by digestion with phospholipase C (*B. cereus*). Individual GPLs (500 μg or less) along with 50 μg of BHT were suspended in 1.5 ml of peroxide-free diethyl ether and 1.5 ml of buffer [17.5 m*M* tris(hydroxy-methyl) aminomethane adjusted to pH 7.3 with HCL, 1.0 m*M* $CaCl_2$] together with 10 units of the enzyme [18]. The mixture was vortex-mixed for 10 s and then shaken at 37°C on a Buchler rotary Evapo-Mix for 2–3 h. The diradylglycerols were extracted with diethyl ether and the extracts passed through a Pasteur pipet containing anhydrous Na_2SO_4. After reduction of volume, the extent of hydrolysis was checked in control samples by spotting the digested lipid in a narrow lane beside one having 25 μg of undigested GPL on a silica gel plate, followed by developing with chloroform–methanol–aqueous ammonia (65:25:4, v/v). The recovered diacylglycerols were converted into TMS ethers.

The TMS ethers of the various diacylglycerols were resolved according to carbon number by GLC on a non-polar capillary column as described above for the TBDMS ethers of the free diacylglycerols. The TMS ethers were resolved into molecular species by GLC on a polar capillary column (15 m × 0.32 mm I.D.) coated with cross-bonded RTx 2330 (Restek Corp., Port Matilda, PA, USA). The carrier gas was hydrogen at 20.7 kPa head pressure and an isothermal column temperature of 250°C [19]. The temperatures of the injector and detector were maintained at 270°C and 300°C, respectively. Determination of major components (greater than 10%) had coefficients of variation (C.V.) of 2% or less, whereas minor components (less than 1%) had a C.V. of *ca.* 10%.

2.8. Analysis of fatty acid methyl esters

The fatty acid methyl esters and dimethyl acetals were determined by capillary GLC using the above polar capillary column and temperature programming [8]. The oven temperature was raised from 100°C to either 130°C or 180°C at 20° C/min and then to 240°C at 5°C/min using hydrogen as the carrier gas at 13.8 kPa head pressure.

2.9. GLC instrumentation

The GLC analyses of the TMS and TBDMS ethers of the diradylglycerols on the non-polar columns were performed with a Hewlett-Packard (Palo Alto, CA, USA) Model 5880 capillary gas chromatograph equipped with a flame-ionization detector and on-column injector. The analyses on the polar columns were done on a Hewlett–Packard Model 5880 capillary gas chromatograph equipped with a flame-ionization detector and a split injector. The peak areas were integrated and recorded by a Hewlett-Packard Series 5880A Level Four Terminal.

2.10. Enzymatic quantitation of sn-1,2-diacylglycerols

The free *sn*-1,2-diacylglycerols (DAGs) were specifically quantitated by diacylglycerol kinase of *E. coli* [20] and the [^{32}P]-phosphatidic acid (PA) formed was isolated by TLC on silica gel H (Analtech, Fisher Scientific) using chloroform–methanol–acetone–acetic acid–water (100:10:40:20:10, v/v) as the developing solvent. Lipids were visualized by autoradiography. In this system phosphatidic acid (R_F 0.70) migrated well ahead of any lysophosphatidic acid and ceramide phosphate (R_F 0.40–0.36), which are also formed by the kinase [21]. The radioactive area corresponding to PA was scraped into glass scintillation vials and radioactivity was measured [1] after chemiluminescence had subsided (usually overnight).

Table 1
Quantitative analysis of 1,2-diacylglycerols in human neuroblastoma (SH-SY5Y) cell extracts

Experimental conditions	*sn*-1,2-DAG (pmol/μg cell protein)	*n*
(A) Control	1.63 ± 0.30	6
	1.94 ± 0.20	9[a]
(B) Plus 10 m*M* LiCl	2.57 ± 0.55	6 $p < 0.05$
(C) Zero time	1.38 ± 0.41	3
(D) Plus 500 pmol DO	3.64 ± 0.38	3 $p < 0.05$
(E) Plus bradykinin	1.75 ± 0.30	3
(F) Serum deprived	1.10 ± 0.12	4
(G) Serum deprived plus 10 m*M* LiCl	1.07 ± 0.12	4

Cells were grown to confluency in 7% fetal calf serum and organic extracts obtained as described in the Experimental section. In series A cells were washed free of culture media and incubated 30 min with HEPES-buffered Krebs–Ringer (KRGH), pH 7.3, 30 min/37°C prior to extraction and analysis. Series B was the same as series A except that the buffer contained 10 m*M* LiCl. Series C included buffer washes and no incubation at 37°C. Series D contained 500 pmol dioleoylglycerol (DO) added to the cell extracts just prior to assay. Series E was the same as series A except that it involved 10 min of incubation with 10 μ*M* bradykinin.

[a] Includes values obtained from series D from which mean value for 500 pmol DO (obtained from standard reference curve) had been substracted.

2.11. Statistics

Data were analyzed for significance by Student's t-tests.

3. Results

Table 1 gives the results of a direct quantitative analysis of the DAGs of human neuroblastoma cells before and after treatment with LiCl. In agreement with Brami et al. [1] we observed a statistically significant ($p < 0.05$) increase (32–58%) in *sn*-1,2-diacylglycerols in these cells after 30 min of incubation with 10 m*M* LiCl when compared to controls incubated with KRGH buffer alone. Addition of 500 pmol of exogenous dioleoylglycerol gave a rise in [^{32}P]phosphatidic acid anticipated from the standard curve of dioleoylglycerol quantitation, which attested to the validity of the measurement.

The increase measured by the kinase provides no information of any change in the composition of the DAGs or about its source.

Table 2 shows that only minimal alterations occurred in the fatty acid composition of the

Table 2
Fatty acid distribution in *sn*-1,2-diacylglycerols and glycerophospholipids of human neuroblastoma cells (SH-SY5Y) incubated for 30 min with or without LiCl in KRGH buffer.

Source of lipid	Fatty acid distribution (mol%)			U/C%[a]
	Saturates	Monoenes	Polyenes	
1,2-DAG	45.7 ± 3.6	20.3 ± 7.5	29.1 ± 3.5	6.5 ± 0.2
1,2-DAG(+Li[a])	46.4 ± 1.9	16.1 ± 7.8	31.3 ± 5.3	6.4 ± 0.3
IGPL + (IGPL + Li^+)	42.5 ± 1.6	17.0 ± 1.5	40.3 ± 1.2	9.0 ± 0.7
CGPL + (CGPL + Li^+)	44.5 ± 1.3	42.6 ± 1.5	10.5 ± 1.5	4.3 ± 0.3

Lipids were purified and derivatized as described in the Experimental section. The numbers are means ± S.D. of replicate GLC analyses of 3–6 experiments as follows: *sn*-1,2-DAG, $n = 3$; *sn*-1,2-DAG (+Li), $n = 3$; IGPL + (IGPL + Li), $n = 6$; CGPL + (CGPL + Li), $n = 6$. All samples were analyzed at least in duplicate. Abbreviations as given in text.

[a] Sum of number of double bonds per chain length × mol% of total fatty acyl chains.

DAGs, when classified as the saturates, monoenes and polyenes. There was a minor relative increase in the content of the 18:0, 20:3w9 and 20:4w6 and a decrease in the ratio of the 16:0 and 18:1 fatty acids with increasing DAG content. However, species of different chain length are not distinguished.

Fig. 1 shows the carbon number distribution of DAGs following incubation of the SH-SY5Y cells with or without LiCl. The elution patterns are closely similar with C_{38} being the major species, which corresponds to a combination of C_{18} and C_{20} fatty acids. Table 3 demonstrates the absence of obvious differences in the carbon number distribution of DAGs between the treated and untreated cell preparations. However, species of different degrees of unsaturation overlap.

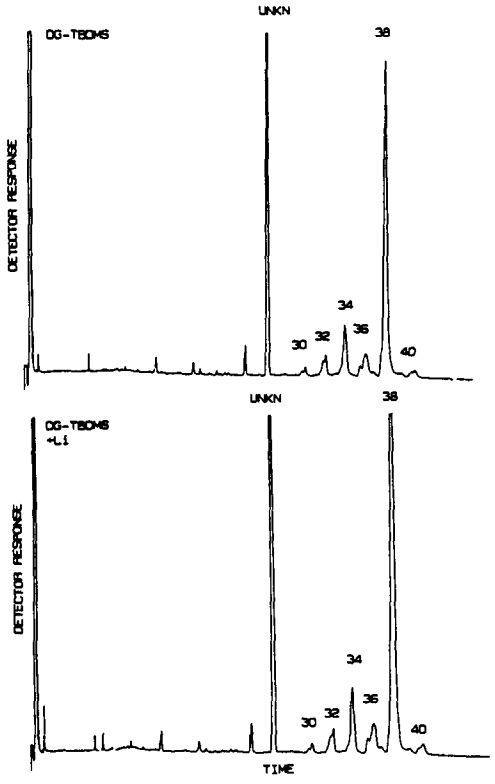

Fig. 1. Carbon number distribution of DAGs following incubation of control (upper panel) and LiCl-treated (lower panel) SH-SY5Y cells as obtained by non-polar capillary GLC of TBDMS ethers. Peaks are identified by total acyl carbon number as given in the figure. Other GLC conditions as given under Experimental.

Fig. 2 compares the molecular species of the DAGs by polar capillary GLC. It can now be seen that the C_{38} peak consists of two components, 18:0–20:3n9 and 18:0–20:4n6, which are present in this cell culture in more or less the same amount. In other culture dishes the amount of the 18:0–20:3n9 component was smaller. Table 4 shows the changes that took place in the molecular species of the DAGs as a result of incubation of the SH-SY5Y cells with LiCl. There was an increase in the major 18:0–20:3n9 and 18:0–20:4n6 species of about 15 and 28 mole%, respectively, and an increase in the minor 18:1–18:1 species of 46 mole% over the control levels. The corresponding decreases occurred largely in the 16:0–16:0 (36%) and 16:0–18:1 (30%) DAG species.

Table 5 gives the DAG composition of the Li^+ treated cells after subtraction of 50% of the total as the background composition due to control. The normalized values of the DAGs released in response to LiCl are compared to those of the *sn*-1,2-diacylglycerol moieties of the IGPLs and CGPLs. Because of the predominance of the polyunsaturated species in both IGPLs and DAGs, IGPLs must be considered the major source of the DAG. However, the increase in the monoenoic species suggests CGPLs as a minor source. Only CGPLs contain these species in abundance. Although EGPLs were also close to the DAGs in their composition of the monoenoic and polyenoic species, it was disqualified as a likely source because of the absence of the alkenylacylglycerol species in DAGs. However, there remained considerable interculture variation, especially in the content of the 18:0–20:3w9 species, which tended to obscure a direct precursor–product relationship.

In order to eliminate inter-culture variation and to obtain a more direct correlation between the composition of DAGs and those of the corresponding IGPLs and CGPLs, we have compared the lipids isolated from the same culture dishes. Fig. 3 shows the polar capillary GLC profiles of the molecular species of the IGPLs and DAGs isolated from a culture accumulating

Table 3
Acyl carbon number distribution of major molecular species from neuroblastoma cells (SH-SY5Y) after incubation with or without LiCl (mol%)

Source of lipid	Acyl carbon number distribution (mol%)						
	C_{28}	C_{30}	C_{32}	C_{34}	C_{36}	C_{38}	C_{40}
1,2-DAG	<0.2	1.4 ± 1.0	7.4 ± 2.7	14.3 ± 5.4	12.9 ± 1.8	60.5 ± 9.1	3.2 ± 0.5
1,2-DAG (+ Li)	<0.2	1.4 ± 0.2	7.2 ± 1.9	12.4 ± 1.0	14.1 ± 6.1	62.5 ± 7.6	2.4 ± 1.1
IGPL + (IGPL + Li)		1.9 ± 0.6	3.7 ± 0.7	4.8 ± 1.1	8.6 ± 0.8	74.5 ± 3.0	6.4 ± 0.6
CGPL + (CGPL + Li)	1.1 ± 0.2	3.1 ± 0.6	17.2 ± 1.0	39.0 ± 1.2	26.8 ± 1.0	8.8 ± 1.9	1.2 ± 0.3

Lipids were purified, derivatized and analyzed by non-polar capillary GLC as described under Experimental. The numbers are means ± S.D. from two or three replicate GLC analyses of three to six experiments as follows: *sn*-1,2-DAG, $n = 3$; *sn*-1,2-DAG, $n = 3$; IGPL + (IGPL + Li), $n = 6$; CGPL + (CGPL + Li), $n = 6$. All samples were analyzed at least in duplicate. Abbreviations as explained in the text.

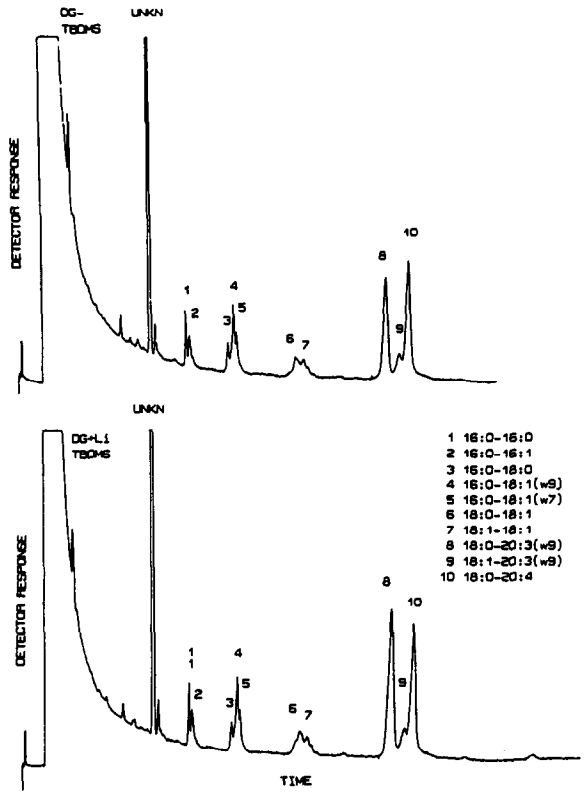

Fig. 2. Molecular species profiles of the DAGs following incubation of control (upper panel) and LiCl-treated (lower panel) SH-SY5Y cells as obtained by polar capillary GLC of TBDMS ethers. Peaks are identified as given in the figure. Other GLC conditions as given under Experimental.

a high amount of 20:3 (n9) acid, which is found mainly in the IGPLs. Identical profiles are seen for the diacylglycerol moieties of the IGPLs and the DAGs, which differ greatly from those of the corresponding CGPLs that did not accumulate 20:3w9 acid [8]. Closely similar profiles were obtained for the diacylglycerol moieties of the IGPLs and the DAGs recovered from a similar cell culture dish following incubation with Li^+ ions (chromatograms not shown). Thus, any increase in the DAGs resulting from LiCl treatment most likely came from hydrolysis of the characteristic species of IGPLs. It was calculated that the diacylglycerol species of CGPLs could have contributed a maximum of 20% of the total DAGs after LiCl treatment.

Fig. 4 shows the effect of LiCl treatment upon cells containing moderate amounts of 20:3n-9 acid upon the molecular species of IGPLs as determined by polar capillary GLC using the TMS ethers. The major species was 18:0–20:4n-6 as in the DAGs (chromatogram not shown). There was no change in the ratio of the molecular species due to incubation with Li^+ ions. The corresponding profile of the diacylglycerol moieties of the CGPLs was characterized by a high proportion of the oligoenoic and low proportion of the polyenoic species, which failed to resemble those of the DAGs (chromatograms not

shown). In this instance, there was a significant increase in the proportion of the 18:1(n9)–18:1(n9) in the DAGs following LiCl treatment, which must have come from CGPL and not from IGPL, since the latter did not contain this species. In such a case, however, the release of

Table 4
Major molecular species of DAG, IGPL and CGPL following incubation of neuroblastoma cells with or without LiCl

Source of Lipids	Major molecular species distribution[a] (mol%)						
	16:0–16:0	16:0–16:1	16:0–18:1n9 (16:0–18:1n7)	18:0–18:1n9 (18:0–18:1n7)	18:1n9–18:1n9 (18:1n9–18:1n7)	16:0–20:4n6 (16:0–20:3n9)	18:0–20:4n6 (18:0–20:3n9)
DAG	3.5 ± 0.6	5.2 ± 0.4	10.4 ± 0.8 (4.5)	1.9 ± 0.2	4.4 ± 0.6	0.4 ± 0.1	32.8 ± 2.1 (16.9 ± 1.7)
DAG (+ Li)	3.8 ± 0.9	3.3 ± 1.3	7.0 ± 0.5 (3.1 ± 0.3)	2.4 ± 1.7	8.2 ± 6.9	0.2 ± 0.2	37.7 ± 2.3 (21.7 ± 1.9)
IGPL + (IGPL + Li)	2.5 ± 0.4	0.3 ± 0.1	1.8 ± 0.2 (1.7 ± 1.0)	3.5 ± 0.6	1.1 ± 0.2	1.7 ± 0.1	55.7 ± 3.0 (6.5 ± 2.0)
CGPL + (CGPL + Li)	7.0 ± 0.9	8.9 ± 0.5	29.5 ± 1.0	7.5 ± 1.4	7.3 ± 1.3 (3.3 ± 0.1)	2.4 ± 1.0	3.3 ± 0.9

Lipids were purified, derivatized and analyzed by polar capillary GLC as described under Experimental. The numbers are means ± S.D. from two or three replicate GLC analyses of two to six experiments as follows: DAG, $n = 2$; DAG + Li, $n = 3$; IGPL + (IGPL + Li), $n = 6$; CGPL + (CGPL + Li), $n = 6$. All samples were analyzed at least in duplicate.
[a] Where applicable, the 18:1n7 and 20:3n9-containing species have been reported separately as bracketed values.

Table 5
Molecular species of DAG released in response to Li^+ incubation in comparison to the *sn*-1,2-diacylglycerol moieties of cellular IGPL and CGPL

Source of Lipids	Major molecular species distribution[a] (mol%)						
	16:0–16:0	16:0–16:1	16:0–18:1n9 (16:0–18:1n7)	18:0–18:1n9 (18:0–18:1n7)	18:1n9–18:1n9 (18:1n9–18:1n7)	16:0–20:4n6 (16:0–20:3n9)	18:0–20:4n6 (18:0–20:3n9)
(DAG + Li)-DAG	4.1 ± 0.9	1.4 ± 1.0	3.6 ± 0.5 (1.7 ± 0.3)	2.9 ± 1.7	12.0 ± 3.6	0.0	42.6 ± 2.3 (26.8 ± 1.9)
IGPL	3.8 ± 0.4	0.3 ± 0.1	1.9 ± 0.2 (1.7 ± 1.0)	4.9 ± 0.2	1.1 ± 0.3	1.7 ± 0.1	52.3 ± 2.9 (5.4 ± 1.4)
CGPL	5.6 ± 1.3	9.3 ± 0.2	30.8 ± 1.6 (trace)	8.2 ± 1.6	7.7 ± 1.4 (3.3 ± 0.1)	2.4 ± 1.1	3.4 ± 1.3

Lipids were purified, derivatized and analyzed by polar capillary GLC as described under Experimental. The numbers are means ± S.D. from two or three replicate GLC analyses of two to four experiments as follows: DAG, $n = 2$; DAG + Li, $n = 3$; IGPL, $n = 4$; CGPL, $n = 4$.
[a] Where applicable, the 18:1n7 and 20:3n9-containing species have been reported separately as bracketed values.

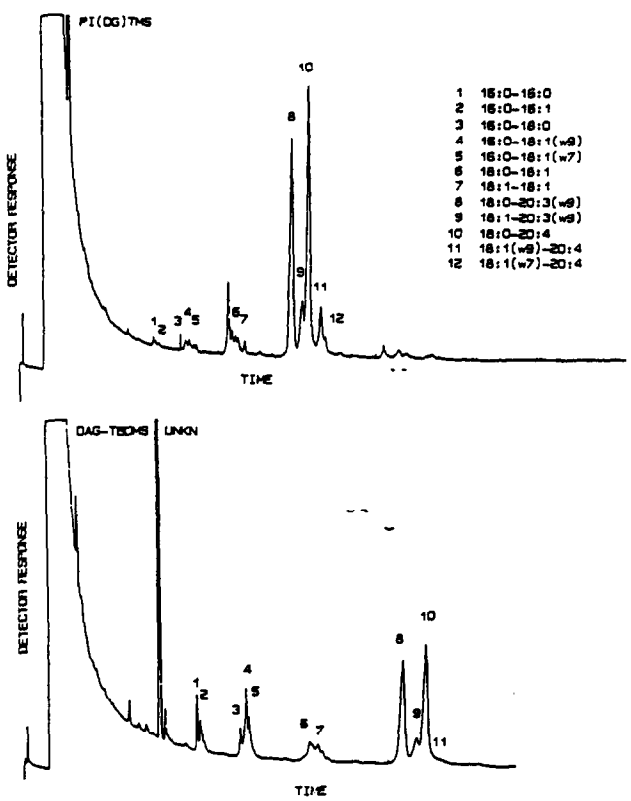

Fig. 3. Molecular species of diacylglycerol moieties of IGPLs (upper panel) and DAGs (lower panel) isolated from the same culture of SH-SY5Y cells as obtained by polar capillary GLC of the TMS and TBDMS ethers, respectively. Other GLC conditions as given under Experimental.

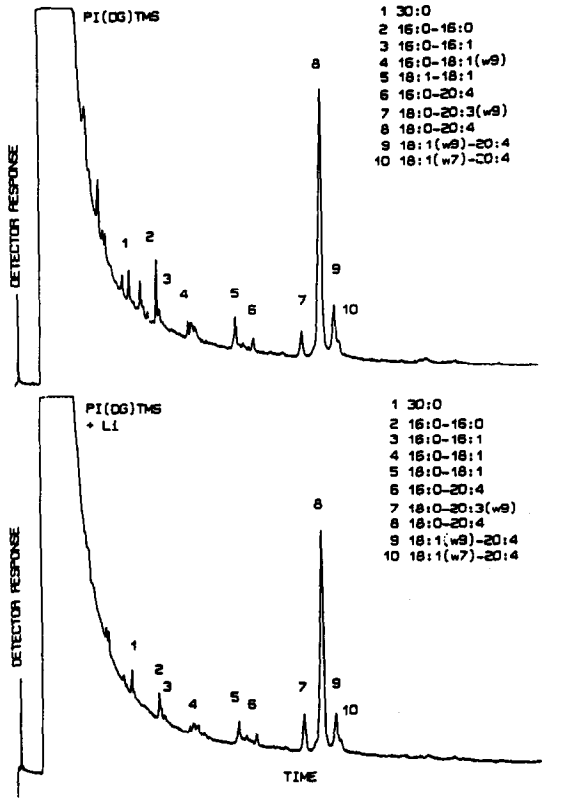

Fig. 4. Molecular species of diacylglycerol moieties of IGPLs of control (upper panel) and LiCl-treated (lower panel) SH-SY5Y cells as obtained by polar capillary GLC of TMS ethers. Peaks are identified as given in the figure. Other GLC conditions as given under Experimental.

the 18:1–18:1 species must have been selective, as CGPL contained the 16:0–18:1(n9) species in a much larger amount, yet its concentration in the DAGs was not increased. The total CGPL contribution to the DAGs was estimated to be 20% at most.

4. Discussion

The inferences made about the precursor–product relationship between the cellular GPLs and the DAGs depend on the analytical strategy. Therefore, each measurement must be qualified.

The kinase is known [20] to be specific for the *sn*-1,2-diacylglycerol enantiomers, although the extent of phosphorylation of the long chain polyunsaturates has not been thoroughly investigated. In comparison to a simple chromatographic measurement of total free diacylglycerol, the kinase values are lower because they exclude the X-1,3-diacylglycerol isomers and any *sn*-2,3-diacylglycerol enantiomers (data not shown). Since the enzyme also reacts with ceramides, the ceramide phosphates must be separated from the phosphatidic acid prior to counting of the radioactivity [1]. We have shown elsewhere [22], that chiral-phase HPLC following conversion of the DAGs into dinitrophenylurethanes can provide an accurate estimate of the quantity of the *sn*-1,2-diacylglycerols in the lipid extract, while on-line mass spectrometry can yield a quantitative assessment of any change in the composition of the diacylglycerol species. In the present

study, we established the change in the molecular species accompanying the increase in diacylglycerol content by determining the composition and molecular association of the fatty acids by GLC before and after LiCl treatment.

The present study confirms the earlier reported [1] increase in diacylglycerol levels caused by Li^+ in SH-SY5Y human neuroblastoma cells and demonstrates that the major source of the extra diacylglycerol are the IGPLs. A corresponding decrease in the polyunsaturated IGPL species could not be shown because of the large difference in pool size. Specific increases in both the 18:0–20:4 and 18:0–20:3 species of DAG were demonstrated, which could have resulted only from hydrolysis of the corresponding IGPL, which contained these species as major components. A smaller increase in the oligoenoic species could have come from CGPLs, because CGPLs contained much more of these species than IGPLs. Hence, it can be calculated that the 32–58% increase in the mass of the diacylglycerols must have been derived largely from the 18:0–20:4 (18:0–20:3) species of IGPL. In view of the known participation of the DAGs as intermediates in phospholipid and triacylglycerol synthesis and hydrolysis, the similarity of the DAG species to those of the IGPLs is remarkable. These findings suggest that LiCl may influence the activity of the IGPL specific phospholipase C, which remains however to be demonstrated.

In earlier studies [1,23] Li^+ appeared to influence intracellular choline metabolism by increasing incorporation of labelled choline into CGPL and sphingomyelin within a 10-min period of incubation. In any event, the present study shows that complementary chromatographic analysis of the molecular species of the diradylglycerols provides a practical method for investigating precursor–product relationships between DAGs and the GPL fractions in cell cultures.

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References

[1] B.A. Brami, U. Leli and G. Hauser, *Biochem. Biophys. Res. Commun.*, 174 (1991) 606.
[2] R.S. Rana and L.E. Hokin, *Physiol. Rev.*, 70 (1990) 115.
[3] M.M. Billah and J.C. Anthes, *Biochem. J.*, 269 (1990) 281.
[4] J.H. Exton, *J. Biol. Chem.*, 265 (1990) 1.
[5] J.M. Besterman, V. Duronio and P. Cutrecassas, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 6785.
[6] M. Pessin, J.G. Altin, M. Jarpe, F. Tansley, R.A. Bradshaw and D.M. Raben, *Cell Regul.*, 2 (1991) 383.
[7] M.S. Pessin, J.J. Baldassare and D.M. Raben, *J. Biol. Chem.*, 265 (1990) 7959.
[8] V.A. Liepkalns, J.J. Myher, A. Kuksis, U. Leli, N. Freysz and G. Hauser, *Biochem. Cell Biol.*, 71 (1993) 141.
[9] R.A. Ross, B.A. Spengler and J.L. Biedler, *J. Natl. Cancer Inst.*, 71 (1983) 741.
[10] V.A. Liepkalns, U. Leli and G. Hauser, *Biol. Psychiatry*, 34 (1993) 51.
[11] W.W. Christie, *Lipid Analysis*, Pergamon Press, Oxford, 1976, pp. 185–189.
[12] K. Abe and K. Kogure, *J. Neurochem.*, 47 (1986) 577.
[13] W.W. Christie, *Lipid Analysis*, Pergamon Press, Oxford, 1976, p. 191.
[14] V.A. Liepkalns, H. Durand and C. Bougeret, *J. Cancer Res. Clin. Oncol.*, 117 (1991) 326.
[15] J.J. Myher and A. Kuksis, *Can. J. Biochem.*, 57 (1979) 117.
[16] J.J. Myher, A. Kuksis, L. Marai and S.K.F. Yeung, *Anal. Chem.*, 50 (1978) 557.
[17] A. Kuksis, J.J. Myher and K. Geher, *J. Lipid Res.*, 34 (1993) 1029.
[18] J.J. Myher, A. Kuksis and S. Pind, *Lipids*, 24 (1989) 396.
[19] A. Kuksis and J.J. Myher, in R.F. Irvine (Editor), *Methods in Inositide Research*, Raven Press, New York, 1990, pp. 187–216.
[20] J. Preiss, C.R. Loomis, W.R. Bishop, R. Stein, J.E. Niedel and R.M. Bell, *J. Biol. Chem.*, 261 (1986) 8597.
[21] J.P. Walsh, *Methods Enzymol.*, 209 (1992) 153.
[22] L.Y. Yeng, A. Kuksis, J.J. Myher and G. Steiner, *J. Lipid Res.*, (1994) submitted for publication.
[23] V.A. Liepkalns, A. Kuksis, G. Hauser, J.J. Myher, U. Leli and N. Freysz, in *Abstracts Amer. Soc. for Cell Biology, 32nd Annual Meeting, November 15–18, 1992*, Denver, CO, Vol. 3, page 146a, Abs. No. 847.