Bombesin stimulates distinct time-dependent changes in the *sn*-1,2-diradylglycerol molecular species profile from Swiss 3T3 fibroblasts as analysed by 3,5-dinitrobenzoyl derivatization and h.p.l.c. separation

Trevor R. PETTITT and Michael J. O. WAKELAM*

Molecular Pharmacology Group, Institute of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

We have developed procedures for the analysis of endogenous diradylglycerol (DRG) molecular species using derivatization with 3,5-dinitrobenzoyl chloride. The introduction of this strong chromophore enabled us to separate less than 1 nmol of DRG into its three classes (diacylglycerol, alkylacylglycerol and alkenylacylglycerol) using a combination of h.p.l.c. and t.l.c. followed by reversed-phase h.p.l.c. to resolve these classes into their component molecular species. When applied to Swiss 3T3 mouse fibroblasts stimulated with bombesin for 25 s, 5 min or 30 min, subtle time-dependent changes in the DRG patterns were observed, with only certain polyunsaturated 1,2-diacylglycerol species [18:0/20:3(*n*–9), 18:0/20:4(*n*–6), 18:0/20:4(*n*–3), 18:0/20:5(*n*–3), 18:1(*n*–9)/20:3(*n*–9), 18:1(*n*–9)/20:4(*n*–6), 16:0/22:6(*n*–3), 18:0/20:3(*n*–6) and 16:0/20:5(*n*–3)] showing significant agonist-stimulated increases. The amounts of the first six species were all raised at 25 s, whereas all except the latter two were elevated at 5 min.

By 30 min these last species were also increased but 18:0/20:3(*n*–9) had returned to basal levels. Overall DRG levels, as measured by total molecular-species peak area, remained effectively constant. No changes in the amount or species profile of 1-alkyl-2-acylglycerol were observed. Comparison of these species with the acyl-chain structure of phospholipids supports the idea that inositol lipids could be the source of DRG at early stimulation times, but phosphatidylcholine appears to be a phospholipase substrate at all times. These results indicate sequential activation of several phospholipases with different substrate specificities and/or access to different phospholipid pools. They also suggest that only polyunsaturated DRGs act as second messengers and that changes in the relative amounts of these species may trigger activation of different proteins and/or isoforms (e.g. the different isoforms of protein kinase C).

INTRODUCTION

Stimulation of cellular events, such as proliferation, by hormones and growth factors involves the generation of various second-messenger molecules, frequently through the hydrolysis of phospholipids. One well-defined pathway involves phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP₂); however, increasing evidence has also implicated other phospholipids, in particular phosphatidylcholine (PtdCho), as alternative sources of lipid-derived second messengers. Of these messengers, the diradylglycerols (DRGs) have been demonstrated to be potent activators of protein kinase C but their exact source (*de novo* synthesis [1,2], hydrolysis of PtdCho [reviewed in 3–6], PtdInsP₂ [reviewed in 7–9], and/or other lipids such as phosphatidylethanolamine (PtdEtn) [10,11]) is still open to debate. However, evidence is mounting that in many cell lines DRG is derived mainly from PtdCho [3–6,12–16], with a smaller contribution from PtdInsP₂, which is mostly generated at short time points (< 60 s) after cell stimulation [12,15,16].

The picture is complicated by different agonists apparently activating different phospholipases which show varying substrate specificities. For instance, both bombesin and platelet-derived growth factor trigger biphasic DRG production in Swiss 3T3 mouse fibroblasts, with an initial burst probably generated by a phosphoinositide-specific phospholipase C [15] and a later, sustained response potentially from a phospholipase D/

phosphatidic acid (PtdOH) phosphohydrolase pathway acting on PtdCho [6,15,17]. However, the phorbol ester, phorbol 12-myristate 13-acetate, only generates a monophasic response [18]. α -Thrombin stimulation of IIC9 Chinese hamster embryo fibroblasts showed a similar biphasic pattern [12], but when pretreated with chymotrypsin before α -thrombin or treated with epidermal growth factor or platelet-derived growth factor, a monophasic production of DRG was observed [13]. The 1,2-diacylglycerol (DAG) molecular-species patterns were very similar for the last three treatments but they all significantly differed from that for α -thrombin alone (at the same time points) and from the patterns for individual cellular phospholipids, thus raising the possibility of different agonist-sensitive phospholipid pools or selective DRG metabolism [13]. Studies on DRG generation in intact resistant arteries also showed agonist-specific changes, with angiotensin II stimulating formation of arachidonate-containing species at 20 s and 5 min, noradrenaline only at 5 min and arginine-vasopressin triggering a decline at both time points [19].

Experiments with rat hepatocytes support the idea of different PtdCho pools, one which is rapidly hydrolysed to DRG and another which is slowly broken down to both DRG and PtdOH when stimulated by Ca²⁺-mobilizing agents such as vasopressin, angiotensin II or calcium ionophore A23187 [14]. Phorbol ester, probably acting via protein kinase C, also induced DRG generation in these cells, but the molecular-species pattern was somewhat different, suggesting the involvement of a different phospho-

Abbreviations used: DRG, diradylglycerol; DAG, diacylglycerol; AAG, 1-alkyl-2-acylglycerol; PtdOH, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; DNB, 3,5-dinitrobenzoyl (derivative); PAF, platelet-activating factor; HBS, Hanks buffered saline.

* To whom correspondence should be addressed.

Figure 1 H.p.l.c. separation of endogenous DRG classes from Swiss 3T3 mouse fibroblasts after DNB derivatization and solid-phase extraction

Conditions: μ Porasil column (5 μ m, 3.9 mm $\times$ 300 mm) with a linear gradient of 100% cyclohexane/hexane/diethyl ether/ethanol (49:49:2:0.1, by vol.) changing to 100% cyclohexane/diethyl ether/ethanol (85:15:0.1, by vol.) in 30 min at 1 ml/min. Detection was at 254 nm. Peak 1 = 1-alkyl-2-acylglycerol + 1-alkenyl-2-acylglycerol; 2 = 1,3-diacylglycerol; 3 = 1,2-diacylglycerol.

lipase C or inhibition of DAG lipase [14] or, alternatively, activation of a phospholipase D/PtdOH phosphohydrolase pathway. Other work such as that on eicosanoid and platelet-activating factor (PAF) generation, where 1-alkyl-2-arachidonyl-PtdCho appears to be preferentially hydrolysed by a phospholipase A_2 , releasing arachidonate for lipoxygenation and/or 1-alkyl-2-lyso-PtdCho for PAF synthesis (reviewed in [20]), supports the concept of specific phospholipid pools and/or molecular-species-specific phospholipases.

Taken together, these findings strongly indicate that different agonists may activate different phospholipase classes and/or isoforms, each with differing substrate specificities (or restricted phospholipid pools due to compartmentalization), resulting in selective hydrolysis of particular phospholipid species. This generates agonist-specific DRG patterns, which may then selectively activate subsequent events (e.g. particular protein kinase C isoforms, since it is already known that they respond differently to phospholipid metabolites such as DAG and non-esterified fatty acid [21]). The cell could thus subtly fine-tune the mechanism for regulating its biological responses.

Owing to technical difficulties in working with very small amounts of lipid material (such as during analysis of molecular species of endogenous DRG), only a few groups have attempted to investigate these possibilities [12–14, 16, 19, 22, 23]. To enable us to do this in a systematic way, we have developed highly sensitive procedures (which we believe are suitable for semiroutine use), based on the 3,5-dinitrobenzoyl chloride derivatization technique of Kito et al. [24], for the h.p.l.c. separation of endogenous DRG classes and their subsequent resolution into molecular species. In this paper we report the methodology used and the changes observed in the diradylglycerol patterns from Swiss 3T3 mouse fibroblasts when stimulated with bombesin for different time periods.

MATERIALS AND METHODS

Materials

All solvents were of AnalaR or h.p.l.c. grade from FSA Lab-

oratory Supplies, Loughborough, Leics., U.K. Diethyl ether was freshly redistilled over KOH. All water was of deionized quality. 3,5-Dinitrobenzoyl chloride was obtained from Aldrich Chemical Co., Poole, Dorset, U.K. DAG, triacylglycerol, fatty acid and fatty acid methyl ester standards were purchased from Sigma Chemical Co., Poole, Dorset, U.K.

Cell culture and stimulation

Swiss 3T3 mouse fibroblasts were cultured in 75 cm² flasks in Dulbecco's modified Eagle's medium containing glutamine (27 mg/ml), penicillin/streptomycin (250 units/ml and 250 μ g/ml respectively), 0.375% (w/v) NaHCO₃ and 10% (v/v) new-born calf serum at 37 °C in a humidified atmosphere of air/CO₂ (19:1, v/v). Cultures were used when cells reached confluency.

Cultures were washed twice with Hanks buffered saline (HBS; 8.00 g/l NaCl, 0.4 g/l KCl, 0.276 g/l CaCl₂·6H₂O, 0.10 g/l MgCl₂·6H₂O, 0.102 g/l MgSO₄·7H₂O, 0.350 g/l NaHCO₃, 0.042 g/l NaH₂PO₄, pH 7.3) and then preincubated in modified HBS [HBS containing 10 mM Hepes, pH 7.3, 10 mM glucose and 1% (w/v) BSA] for 20 min at 37 °C. After removal of the medium, the cells were stimulated by incubation with 100 nM bombesin in modified HBS for 0, 25 s, 5 min or 30 min, at 37 °C. Incubations were stopped by aspiration of the medium followed by immediate addition of ice-cold methanol.

Cells were scraped from the flasks, transferred to glass screw-capped tubes and the lipids extracted using the Folch-wash procedure [25]. Briefly, chloroform was added to give a chloroform/methanol ratio of 2:1 (v/v) followed by sufficient 0.88% (w/v) KCl to give a chloroform/methanol/water ratio of 2:1:1 (by vol.). After vigorous mixing, the phases were allowed to separate, the upper phase was discarded and the lower organic phase washed with methanol/0.88% (w/v) KCl (1:1, v/v). After being dried under a stream of nitrogen, the sample was resuspended in 1 ml chloroform and stored in a sealed tube at –20 °C until required.

H.p.l.c. separation of phospholipids

Total lipid extracts were resuspended in 20 μ l of propan-2-ol/acetonitrile/methanol/1 mM tetrabutylammonium phosphate, pH 7.4 (60:20:10:9.5, by vol.) and separated isocratically on a Hypersil-APS column (5 μ m; 4.6 mm $\times$ 250 mm; FSA) using the same solvent at 1 ml/min with detection at 206 nm.

Phospholipase C hydrolysis

Dried total lipid extracts or individual phospholipids collected from h.p.l.c. were resuspended in 25 μ l of diethyl ether and sonicated with 1.25 ml of incubation buffer (0.2 M potassium phosphate, 1 mM 2-mercaptoethanol, 0.5 mM ZnCl₂, 5 mM H₃BO₃, pH 7.0). Hydrolysis was achieved by incubation with 50 units of phospholipase C from *Bacillus cereus* plus 1 unit from *Bacillus thuringiensis* for 4 h at 37 °C in a shaking water-bath. The generated DRGs were extracted with 3 $\times$ 2 ml diethyl ether.

Sample derivatization

This was modified from Kito et al. [24,26]. Briefly, 0.5 ml of pyridine (dried over NaOH for 30 min before use) containing 25 mg of 3,5-dinitrobenzoyl chloride was added to each sample, the tube sealed and heated at 60 °C for 15 min. The reaction was stopped by the addition of 2 ml of methanol/water (4:1, v/v)

Table 1 Relative retention times of individual diradylglycerol-DNB species

Retention times are relative to 1,2-12:0/12:0-DNB as separated on Spherisorb S50DS2 Excel or Lichrosorb RP-18 using a gradient of acetonitrile/propan-2-ol (9:1, v/v) changing to (1:1, v/v) in 45 min at 1 ml/min and detection at 254 nm. Peak numbers refer to peak labelling in Figures 2, 4 and 5 indicating 1,2-DAG and AAG species detected in 3T3 fibroblasts.

Molecular species*	Peak no.	1,2-DAG		Peak no.	Alkylacylglycerol S50DS2
		S50DS2	Lichrosorb RP-18		
18:1(<i>n</i> -9)/20:5(<i>n</i> -3)	1	1.58	1.57		2.04
16:0/20:5(<i>n</i> -3)	2	1.64	1.68		2.13
16:0/20:4(<i>n</i> -3)		1.64	1.71		—
18:1(<i>n</i> -9)/22:6(<i>n</i> -3)	3	1.71	1.71		2.13
18:2(<i>n</i> -6)/18:2(<i>n</i> -6)	3	1.71	1.71		—
16:1/18:2(<i>n</i> -6)		1.71	1.79		—
16:0/22:6(<i>n</i> -3)	4	1.76	1.79		—
14:0/18:2(<i>n</i> -6)		1.76	1.83		—
16:1/16:1	5	1.81	1.83		—
14:0/16:1	5	1.81	1.87		2.35
18:1(<i>n</i> -9)/22:5(<i>n</i> -3)	6	1.87	1.92		—
18:1(<i>n</i> -9)/20:4(<i>n</i> -6)	7	1.92	1.95		2.40
16:0/22:5(<i>n</i> -3)		1.92	2.02		—
14:0/20:3(<i>n</i> -9)		1.92	2.07		2.40
14:0/14:0		1.92	2.07		2.43
16:0/20:4(<i>n</i> -6)	8	1.98	2.07		2.49
20:3/20:3	9	2.04	2.13		—
18:0/20:5(<i>n</i> -3)	10	2.09	2.24		2.59
18:0/20:4(<i>n</i> -3)	11	2.12	2.24		—
18:1(<i>n</i> -9)/18:2(<i>n</i> -6)	12	2.15	2.24	1	2.64
16:1/18:1(<i>n</i> -9)	12	2.15	2.24		2.64
14:0/18:1(<i>n</i> -9)	13	2.20	2.30	2	2.73
18:1(<i>n</i> -9)/20:3(<i>n</i> -6)		2.20	2.30		—
16:0/22:4(<i>n</i> -6)		2.20	2.37		—
16:0/18:2(<i>n</i> -6)		2.20	2.37		2.73
16:0/16:1	13	2.20	2.37	2	2.73
14:0/16:0	14	2.28	2.37	3	2.81
16:0/20:3(<i>n</i> -6)		2.28	2.37		—
18:0/18:3(<i>n</i> -6)		2.32	2.37		—
18:1(<i>n</i> -9)/20:3(<i>n</i> -9)	15	2.32	2.37	4	2.81
16:0/20:3(<i>n</i> -9)	16	2.39	2.47	5	2.91
18:0/22:5(<i>n</i> -3)	16	2.39	2.55		2.91
18:0/20:4(<i>n</i> -6)	17	2.45	2.60	5	2.94
15:0/15:0	18	2.52	2.69		—
18:1(<i>n</i> -9)/18:1(<i>n</i> -9)	19	2.60	2.73	6	3.08
16:0/18:1(<i>n</i> -9)	20	2.67	2.88	7/8†	3.16
18:0/18:2(<i>n</i> -6)	20	2.67	2.88		—
18:0/16:1		2.67	2.88		—
18:0/20:3(<i>n</i> -6)	21	2.71	2.88		—
16:0/18:1(<i>n</i> -7)		2.71	3.00		—
14:0/18:0		2.76	3.00		3.26
16:0/16:0	22	2.76	3.07	9	3.26
18:0/20:3(<i>n</i> -9)	23	2.85	3.07	10	3.36
17:0/18:1(<i>n</i> -9)	24	2.90	3.18	11	3.41
16:0/17:0	25	3.00	3.38		—
18:1(<i>n</i> -9)/20:1(<i>n</i> -9)	26	3.03	3.31	12	3.50
16:0/20:1(<i>n</i> -9)	27	3.10	3.38	13	3.60
18:0/18:1(<i>n</i> -9)	28	3.13	3.44	14	3.63
18:0/18:1(<i>n</i> -7)		3.22	3.58		3.63
16:0/18:0	29	3.22	3.66	15	3.71
18:0/20:1(<i>n</i> -9)		3.54	4.00		4.01
18:0/18:0	30	3.67	4.28	16	4.14

* Where the double bond (*n*-) designation is not given, the exact fatty acid positional isomer was not determined.

† Peak 7 is 1-*O*-18:1/16:0 and peak 8 is 1-*O*-16:0/18:1(*n*-9).

followed by 2 ml of water. This was passed on to a C₁₈ Sep-Pak mini-column [Waters-Millipore; previously washed sequentially with 10 ml of diethyl ether, 15 ml of methanol and 15 ml of methanol/water (4:1, v/v)], washed with 2 × 15 ml of methanol/water (4:1, v/v) and finally eluted with 25 ml of freshly redistilled diethyl ether. After being dried under nitrogen, the eluate was

resuspended in a small volume of diethyl ether and stored at -20 °C.

H.p.l.c. separation of diradyl classes

The dinitrobenzoylated (DNB) material was resuspended in

20 μ l of cyclohexane/diethyl ether/ethanol (85:15:0.1, by vol.) and separated on a μ Porasil column (5 μ m, 3.9 mm $\times$ 300 mm; Waters-Millipore) using a linear solvent gradient changing from 100% hexane/cyclohexane/diethyl ether/ethanol (49:49:2:0.1, by vol.) to 100% cyclohexane/diethyl ether/ethanol (85:15:0.1, by vol.) in 30 min at 1 ml/min with detection at 254 nm. Peaks corresponding to 1,2-DAG-DNB and 1-alkenyl-2-acylglycerol-DNB (AAG-DNB) + 1-alkenyl-2-acylglycerol-DNB (incomplete resolution of these two classes) were collected.

T.l.c. separation of alkylacyl and alkenylacyl classes

Peaks collected from h.p.l.c. were spotted on activated silica-gel G60 t.l.c. plates containing fluorescent indicator F₂₅₄ (20 cm $\times$ 20 cm; Merck) and separated using hexane/diethyl ether (13:7, v/v). When viewed under u.v. (254 nm), the DNB derivatives appear as dark spots on a green background. Those corresponding to AAG-DNB (R_F 0.43) and 1-alkenyl-2-acylglycerol-DNB (R_F 0.37) were scraped into sintered funnels and eluted with 20 ml of freshly redistilled diethyl ether. The R_F values for DNB-derivatized 1,3- and 1,2-DAGs were 0.33 and 0.29 respectively.

H.p.l.c. separation of molecular species

A known amount of previously prepared 1,2-12:0/12:0-DNB was added to each DRG-DNB class as internal standard before drying under a stream of nitrogen and resuspension in 20 μ l of acetonitrile/propan-2-ol (1:1, v/v). Separation of molecular species was achieved on a Spherisorb S50DS2 Excel column (5 μ m, 4.6 mm $\times$ 250 mm; Hichrom Ltd., Reading, Berks., U.K.) or a LiChrosorb RP-18 column (5 μ m, 4 mm $\times$ 250 mm; Merck) using a linear gradient of 100% acetonitrile/propan-2-ol (9:1, v/v) changing to 100% acetonitrile/propan-2-ol (1:1, v/v) in 45 min at 1 ml/min with detection at 254 nm.

G.l.c. identification of molecular species

Individual molecular species collected from h.p.l.c. [dried under nitrogen with 500 ng of heneicosanoic acid (21:0) and/or 1000 ng of tripentadecanoin (15:0) as internal standard] were trans-methylated in sealed tubes by refluxing with 2 ml of 2.5% (v/v) H₂SO₄ in dry methanol at 70 °C for 2 h. The reaction was stopped by addition of 5 ml of 5% (w/v) NaCl and fatty acid methyl esters (+ dimethyl acetals from 1-alkenyl-2-acylglycerol) extracted with 3 $\times$ 3 ml of redistilled hexane. These were separated on an SPB-1 capillary column (10 μ m film thickness; 30 m $\times$ 0.25 mm internal diam.; Supelco) using a temperature gradient of 160–240 °C rising at 1 °C/min, a split ratio of 20:1 and a gas flow rate of 0.5 ml/min. Identification was by reference to authentic standards.

Other techniques

For radiolabelling experiments, 0.25 μ Ci of [¹⁴C]1,2-18:0/20:4(*n*–6) was added to the total lipid extract before derivatization. Fractions were taken for scintillation counting at each stage.

Total 1,2-DRG measurements were made using the DAG kinase assay of Preiss et al. [27] as modified by Paterson et al. [28].

RESULTS

Starting from the conditions developed by Kito and co-workers [24,26], we modified procedures to enable the derivatization, separation and identification of very small amounts of endogenous DRG (< 1 nmol total DRG; sufficient material was obtained from one 75 cm² flask of confluent Swiss 3T3 mouse fibroblasts) in the presence of other lipophilic components, without the requirement for initial t.l.c. separation or the use of a radiolabel. Difficulties arose from the presence of what would normally be very minor contaminants in the solvents and other materials used, since these often gave spurious peaks on the h.p.l.c. profiles at the high sensitivity required for detection of individual molecular species. T.l.c. was initially employed for the separation and clean-up of diradylglycerol-DNB derivatives, but it became apparent that other components still contaminated these diradyl bands. No t.l.c. system appeared capable of cleanly resolving the components of interest so an h.p.l.c. procedure was developed. This has the advantage that the sample remains in solution, thus minimizing the risk of oxidation and other rearrangements which can cause problems with t.l.c.

H.p.l.c. using a μ Porasil (silica) column with a gradient of 100% hexane/cyclohexane/diethyl ether/ethanol (49:49:2:0.1, by vol.) changing to 100% cyclohexane/diethyl ether/ethanol (85:15:0.1, by vol.) in 30 min was found to clearly separate 1,2- and 1,3-DAG-DNBs from other components but it only partially resolved AAG-DNB from 1-alkenyl-2-acylglycerol-DNB (Figure 1). A subsequent t.l.c. separation, with a solvent system of hexane/diethyl ether (13:7, v/v), does fully resolve this pair; however, it was found that 3T3 mouse fibroblasts produce very little, or no, 1-alkenyl-2-acylglycerol under basal or stimulated conditions (less than 1% of total DRG), hence this h.p.l.c. peak was assumed to be solely AAG for all subsequent analyses of endogenous DRG from these cells. The large peak just ahead of AAG-DNB (Figure 1) is a contaminant from the derivatization procedure which co-chromatographs with 1,2-DAG-DNB in most t.l.c. systems tested. Different molecular species within a particular diradyl class had minimal effect on peak mobility (on μ Porasil), although 1,2-12:0/12:0-DNB did run towards the tail

Figure 2 H.p.l.c. separation of 1,2-DAG-DNB molecular species from Swiss 3T3 mouse fibroblasts

Conditions: Spherisorb S50DS2 Excel column (5 μ m, 4.6 mm $\times$ 250 mm) with a linear gradient of 100% acetonitrile/propan-2-ol (9:1, v/v) to 100% acetonitrile/propan-2-ol (1:1, v/v) in 45 min at 1 ml/min. Detection was at 254 nm. 1,2-12:0/12:0-DNB was added as internal standard. See Table 1 for identity of numbered peaks.

Table 2 Bombesin-stimulated changes in 1,2-DAG molecular-species profiles

Swiss 3T3 mouse fibroblasts were stimulated with 100 nM bombesin for 0, 25 s, 5 min or 30 min. Values are percentages $\pm$ S.D. (where $n = 4$). tr = trace ($< 0.05\%$). Peak numbers correspond to peak labelling in Figure 2. $P < 0.005$ compared with controls (Student's t test).

Peak no.	Molecular species	Composition (%)			
		Control	Bombesin stimulation		
			25 s	5 min	30 min
1	18:1($n-9$)/20:5($n-3$)	0.3 $\pm$ 0.2	0.2 $\pm$ 0.1	0.2 $\pm$ tr	0.4 $\pm$ tr
2	16:0/20:5($n-3$)	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1	1.2 $\pm$ 0.3*
3	18:2($n-6$)/18:2($n-6$); 18:1($n-9$)/22:6($n-3$)	0.4 $\pm$ 0.1	0.3 $\pm$ 0.2	0.2 $\pm$ tr	0.5 $\pm$ 0.2
4	16:0/22:6($n-3$)	0.4 $\pm$ 0.1	0.4 $\pm$ 0.2	0.9 $\pm$ 0.4*	1.4 $\pm$ 0.3*
5	16:1/16:1; 14:0/16:1	1.4 $\pm$ 0.3	1.1 $\pm$ 0.2	1.4 $\pm$ 0.6	1.3 $\pm$ tr
6	18:1($n-9$)/22:5($n-3$)	1.4 $\pm$ 0.2	1.4 $\pm$ 0.1	1.6 $\pm$ 0.4	1.4 $\pm$ 0.1
7	18:1($n-9$)/20:4($n-6$)	1.1 $\pm$ 0.2	1.5 $\pm$ 0.3*	1.6 $\pm$ 0.2*	1.8 $\pm$ 0.4*
8	16:0/20:4($n-6$)	0.3 $\pm$ 0.1	0.4 $\pm$ 0.3	0.4 $\pm$ 0.2	0.5 $\pm$ 0.1
9	20:3/20:3	0.1 $\pm$ tr	0.2 $\pm$ 0.1	0.1 $\pm$ tr	0.2 $\pm$ 0.1
10	18:0/20:5($n-3$)	0.6 $\pm$ 0.4	1.4 $\pm$ 0.3*	1.1 $\pm$ 0.2*	3.3 $\pm$ 0.8*
11	18:0/20:4($n-3$)	tr	0.9 $\pm$ 0.7*	1.5 $\pm$ 0.9*	2.1 $\pm$ tr*
12	18:1($n-9$)/18:2($n-6$); 16:1/18:1($n-9$)	3.1 $\pm$ 0.5	2.6 $\pm$ 0.3	2.9 $\pm$ 0.3	2.9 $\pm$ 0.2
13	16:1/16:1; 14:0/18:1($n-9$)	12.0 $\pm$ 0.8	10.5 $\pm$ 1.4	10.6 $\pm$ 1.2	10.4 $\pm$ 1.2
14	14:0/16:0	7.4 $\pm$ 0.7	5.9 $\pm$ 0.3	6.2 $\pm$ 1.1	4.7 $\pm$ 1.0
15	18:1($n-9$)/20:3($n-9$)	0.5 $\pm$ 0.1	3.6 $\pm$ 2.4*	3.1 $\pm$ 0.9*	1.9 $\pm$ 0.7*
16	18:0/20:3($n-9$); 18:0/22:5($n-3$)	1.0 $\pm$ 0.3	1.4 $\pm$ 0.5	1.1 $\pm$ 0.1	1.3 $\pm$ 0.2
17	18:0/20:4($n-6$)	2.4 $\pm$ 0.7	4.0 $\pm$ 1.0*	3.9 $\pm$ 0.4*	4.5 $\pm$ 0.9*
18	15:0/15:0	0.5 $\pm$ tr	0.4 $\pm$ 0.1	0.6 $\pm$ 0.2	0.3 $\pm$ 0.1
19	18:1($n-9$)/18:1($n-9$)	6.6 $\pm$ 0.5	6.8 $\pm$ 0.7	7.1 $\pm$ 0.7	6.7 $\pm$ 0.2
20	16:0/18:1($n-9$); 18:0/18:2($n-6$)	24.4 $\pm$ 1.0	21.3 $\pm$ 2.4	20.8 $\pm$ 1.1	21.6 $\pm$ 1.8
21	18:0/20:3($n-6$)	0.6 $\pm$ 0.2	0.6 $\pm$ 0.1	0.6 $\pm$ 0.2	1.2 $\pm$ 0.3*
22	16:0/16:0	11.0 $\pm$ 0.6	9.6 $\pm$ 1.0	8.9 $\pm$ 0.8	7.4 $\pm$ 1.6
23	18:0/20:3($n-9$)	4.1 $\pm$ 0.8	9.7 $\pm$ 3.9*	8.7 $\pm$ 1.1*	4.3 $\pm$ 0.4
24	17:0/18:1($n-9$)	0.5 $\pm$ 0.1	0.4 $\pm$ tr	0.5 $\pm$ 0.2	0.5 $\pm$ 0.1
25	16:0/17:0	0.4 $\pm$ 0.2	0.3 $\pm$ 0.1	0.3 $\pm$ 0.2	0.3 $\pm$ 0.1
26	18:1($n-9$)/20:1($n-9$)	1.0 $\pm$ 0.3	0.5 $\pm$ 0.2	0.6 $\pm$ 0.3	0.5 $\pm$ 0.1
27	16:0/20:1($n-9$)	0.9 $\pm$ 0.1	0.7 $\pm$ 0.1	0.8 $\pm$ 0.1	0.7 $\pm$ 0.1
28	18:0/18:1($n-9$)	5.7 $\pm$ 0.4	4.9 $\pm$ 0.8	4.7 $\pm$ 0.5	4.5 $\pm$ 0.2
29	16:0/18:0	7.2 $\pm$ 0.7	6.0 $\pm$ 0.8	4.7 $\pm$ 0.8	7.1 $\pm$ 0.7
30	18:0/18:0	1.1 $\pm$ 0.3	0.6 $\pm$ 0.2	0.7 $\pm$ 0.2	0.9 $\pm$ 0.1

of the total 1,2-diacyl peak, whereas 1,2-20:0/20:0-DNB ran towards the leading edge.

Initial experiments where samples were spiked with [^{14}C]1,2-18:0/20:4($n-6$) and the radiolabel distribution was followed throughout the derivatization and separation procedures showed that about 50% of the starting material was recovered in the diethyl ether eluate from the Sep-Pak. Nearly all the rest came off in the methanol/water (4:1, v/v) wash, possibly as underivatized DAG. Altering the wash ratio to 7:3 (v/v) slightly increased the recovery in the diethyl ether eluate, but also increased the amount of radiolabelled material sticking to the silica column in the subsequent h.p.l.c. step which could only be removed by washing with a more polar solvent system such as acetonitrile/propan-2-ol (1:1, v/v). Some 50–60% of the [^{14}C]-labelled material injected on to the μ Porasil h.p.l.c. column was recovered in the 1,2-DAG-DNB peak, 5% as other minor peaks, 5% as a very broad band eluted after 1,2-DAG-DNB and most of the rest as more polar material which was not eluted within 60 min. Overall recovery of [^{14}C]1,2-18:0/20:4($n-6$) as the DNB derivative was 25–30%. Careful drying of the pyridine immediately before use

gave the best recoveries since even very small amounts of moisture decreased the derivatization efficiency.

Separation of individual DRG classes on a reversed-phase C_{18} column was unable to fully resolve all the molecular species (over 30 are present in 1,2-DAG from 3T3 fibroblasts) but, as different C_{18} columns have slightly different retention characteristics, most species can be identified when two columns are used (e.g. Lichrosorb RP-18 and Spherisorb S5ODS2). Direct fatty acid analysis of each molecular-species peak from endogenous DRG samples was normally impossible because of the very small amount of material (< 100 ng per peak). However, by hydrolysing a total 3T3 fibroblast lipid extract (from 10×75 cm 2 flasks of confluent cells) with phospholipase C to generate a total DRG pattern and then derivatizing it, sufficient material was available to collect individual molecular-species peaks, transmethyrate them and analyse by capillary g.l.c. The h.p.l.c. retention times of these species, relative to 1,2-12:0/12:0-DNB internal standard (Table 1), could then be used to identify the molecular species peaks from endogenous DRG samples.

When these procedures were applied to Swiss 3T3 mouse

Figure 3 Changes in DAG pattern in response to bombesin stimulation

The figure illustrates the DAG species that change in response to stimulation of 3T3 cells with bombesin (100 nM). Other details are as in the legend to Table 2.

Figure 4 H.p.l.c. separation of AAG-DNB molecular species from Swiss 3T3 mouse fibroblasts

Conditions were as in Figure 2. See Table 1 for identity of numbered peaks.

fibroblast samples, the predominant 1,2-DAG species were found to be saturated and monounsaturated, with the major form being 16:0/18:1(*n*-9). This co-eluted with a smaller amount of 18:0/18:2(*n*-6) on both h.p.l.c. columns used (Figure 2). Other quantitatively important forms include 18:0/18:1(*n*-9) (peak 28), 16:0/18:0 (peak 29) and 16:0/16:0 (peak 22). Polyunsaturated DAG species such as 18:0/20:4(*n*-6) (peak 17) and 16:0/20:5(*n*-3) (peak 2) are only relatively minor components in resting cells.

Bombesin stimulation of these cells for 25 s, 5 min or 30 min generated subtle changes in the 1,2-DAG pattern. Whereas the relative amounts of most species remained effectively unchanged, those of nine polyunsaturated species were elevated significantly relative to the controls (Table 2). These were 18:0/20:3(*n*-9),

18:0/20:4(*n*-6), 18:0/20:4(*n*-3), 18:0/20:5(*n*-3), 18:1(*n*-9)/20:3(*n*-9), 18:1(*n*-9)/20:4(*n*-6), 16:0/22:6(*n*-3), 18:0/20:3(*n*-6) and 16:0/20:5(*n*-3). The first six species were all increased at 25 s, while all except the latter two were increased at 5 min. By 30 min these last species were also elevated, but 18:0/20:3(*n*-9) had returned to basal levels (see Figure 3).

Total 1,2-DRG measurements using the DAG kinase assay indicated increased DRG levels after bombesin stimulation as previously reported [18], although no corresponding mass increases were seen with the DNB-derivatized/h.p.l.c.-separated material, as measured by total peak area relative to the 1,2-12:0/12:0 internal standard, indicating that small falls in the levels of saturated and monounsaturated species balanced the rise in polyunsaturated species.

No changes were observed in the AAG pattern (Figure 4) at any time point although this represented 28–34% of the total 1,2-DRG. The total amount of this DRG class was unaffected by bombesin stimulation as measured by total peak area. The major AAG species are 16:0/18:0 (peak 15), 16:0/16:0 (peak 9), 18:1/16:0 (peak 7) and 14:0/18:1(*n*-9) (peak 2).

Phospholipase C hydrolysis of Swiss 3T3 mouse fibroblast PtdCho, PtdEtn, PtdIns, and phosphatidylserine (PtdSer), clearly separated by h.p.l.c. on a Hypersil-APS column, followed by DNB derivatization and h.p.l.c. resolution of the resultant DRG species gave molecular-species profiles as shown in Figure 5. The 1,2-diacyl-PtdCho pattern (Figure 5a) is very similar to that for endogenous 1,2-DAG from resting cells (compare with Figure 2), with the major species being 14:0/18:1(*n*-9) + 16:0/16:1, 16:0/18:1(*n*-9) + 18:0/18:2(*n*-6), 16:0/16:0, 18:1(*n*-9)/18:1(*n*-9) and 18:0/18:1(*n*-9). One of the few main differences is that 16:0/18:0 (peak 29) is relatively less important in PtdCho, whereas 18:0/18:1(*n*-9) (peak 28) assumes a greater importance. Likewise, the endogenous AAG pattern is very similar to that from 1-alkyl-2-acyl-PtdCho (results not shown). The PtdIns profile (Figure 5b) is rich in 18:0/20:3(*n*-9), 18:0/20:4(*n*-6), 18:0/20:5(*n*-3), 18:1(*n*-9)/20:3(*n*-9) and 18:1(*n*-9)/20:4(*n*-6), the same species that increase signi-

Figure 5 Molecular-species profiles for Swiss 3T3 mouse fibroblast phospholipids

Profiles are shown for PtdCho (a), PtdIns (b), PtdEtn (c) and PtdSer (d) obtained after phospholipase C hydrolysis and DNB derivatization of the resultant DRGs. H.p.l.c. separation and peak numbering are as in Figure 2.

ificantly (at early time points) in 1,2-DAG from bombesin-stimulated fibroblasts. The PtdEtn profile (Figure 5c) is also relatively rich in these species, together with 18:0/18:1(*n*-9), 16:0/18:1(*n*-9) + 18:0/18:2(*n*-6) and 18:1(*n*-9)/18:1(*n*-9). PtdSer, the profile (Figure 5d) of which is particularly rich in 18:0/18:1(*n*-9), shows no obvious similarities to that for endogenous DAG. The relative proportions of the different diacyl molecular species in these phospholipids are shown in Table 3.

DISCUSSION

The DNB-derivatization procedures used here for the analysis of endogenous DRG species are highly sensitive (< 10 ng of each molecular species can be detected after attachment of a 3,5-dinitrobenzoyl-chromatophore) and suitable for routine analysis. Elimination of an initial t.l.c. lipid separation decreases the risk of oxidation and/or molecular rearrangement, while direct derivatization of the total lipid extract decreases losses and speeds up the procedure, although possibly at the expense of reduced (25–30%) derivatization efficiency. H.p.l.c. purification of the DRG classes, although slower than t.l.c. if four or more samples are separated, gives a much cleaner product, thus allowing smaller samples to be used without the increased risk of contaminants interfering with the final reversed-phase h.p.l.c. separation. Further modifications may enable h.p.l.c. resolution of AAG-DNB from 1-alkenyl-2-acylglycerol-DNB, although the insignificant amounts of the latter detected in 3T3 fibroblasts may diminish the need for this in similar applications.

The finding that, whereas total DRG measurements increased slightly after bombesin stimulation, as determined by DAG kinase assay (see also [18]), no corresponding increase was observed on quantification by total molecular-species peak area, suggests that the kinase assay does not phosphorylate all DRG species with the same efficiency. This partial selectivity is probably

for the polyunsaturated forms which change most after cell stimulation, hence giving an exaggerated mass change. This is perhaps not surprising since all enzymes show some degree of substrate selectivity, even when presented with very closely related structures. Further support for this conclusion comes from our observations that the t.l.c. mobility of the PtdOH product from the DAG kinase assay is closer to that seen with standard longer-chain polyunsaturated species rather than shorter-chain more-saturated species. The use of 18:0/20:4(*n*-6) as calibration standard for the DAG kinase assay means that the assay is most accurately quantifying changes in the polyunsaturated forms, particularly 18:0/20:4(*n*-6). Standardization against a saturated or monounsaturated form may decrease the measured response since these species show smaller relative changes.

When compared with the molecular-species profiles for individual phospholipids, the 1,2-DAG pattern observed in quiescent 3T3 fibroblasts (Figure 2 and Table 2) has many similarities to that for total cellular PtdCho (Figure 5a and Table 3), although 18:0/18:1(*n*-9), 18:0/22:5(*n*-3), 16:0/20:3(*n*-9), 16:0/20:4(*n*-6) and 16:0/20:5(*n*-3) are proportionately less important in this phospholipid. Whereas stimulation enhances the proportions of some of these, others remain largely unaffected even after a 30 min exposure to bombesin when most DRG is thought to be derived from PtdCho [3–6,10–14]. These results contradict ideas of a non-specific conversion of PtdCho into 1,2-DRG, since elevation of all species found in the parent phospholipid would be expected. In particular, 14:0/18:1(*n*-9) + 16:0/16:1, 18:0/18:1(*n*-9), 16:0/16:0, 16:0/18:1(*n*-9) + 18:0/18:2(*n*-6) and 18:1(*n*-9)/18:1(*n*-9) should increase since these are the predominant forms in material derived from PtdCho. However, if anything, a slight decrease is observed after bombesin stimulation, even at 30 min. This may be explained if compartmentalization limits the access of a phospholipase to PtdCho, or if the enzyme is selectively

Table 3 Diacyl structures of the major phospholipids in Swiss 3T3 mouse fibroblasts

Percentage compositions are shown of the major 1,2-diacyl molecular species from PtdCho, PtdEtn, PtdIns and phosphatidylserine (PtdSer). Peak numbers correspond to peak labelling in Figures 2 and 5. tr = trace (< 0.05%).

Peak no.	1,2-Diacyl molecular species	Composition (%)			
		PtdCho	PtdEtn	PtdIns	PtdSer
1	18:1(<i>n</i> -9)/20:5(<i>n</i> -3)	0.6	2.1	1.0	0.1
2	16:0/20:5(<i>n</i> -3)	0.8	1.0	0.6	0.1
3	18:2(<i>n</i> -6)/18:2(<i>n</i> -6), 18:1(<i>n</i> -9)/22:6(<i>n</i> -3)	0.3	0.4	0.2	0.3
4	16:0/22:6(<i>n</i> -3)	1.2	0.4	0.2	tr
5	16:1/16:1, 14:0/16:1	1.0	0.3	0.7	0.4
6	18:1(<i>n</i> -9)/22:5(<i>n</i> -3)	0.6	1.4	0.5	0.2
7	18:1(<i>n</i> -9)/20:4(<i>n</i> -6)	2.1	3.3	5.5	0.6
8	16:0/20:4(<i>n</i> -6)	1.6	1.4	1.0	0.1
9	20:3/20:3	0.6	0.1	2.6	0.1
10	18:0/20:5(<i>n</i> -3)	1.0	6.3	4.9	2.0
11	18:0/20:4(<i>n</i> -3)	0.3	0.1	3.1	tr
12	18:1(<i>n</i> -9)/18:2(<i>n</i> -6), 16:1/18:1(<i>n</i> -9)	3.4	1.8	1.0	0.4
13	14:0/18:1(<i>n</i> -9), 16:0/16:1	11.4	3.3	1.0	4.6
14	14:0/16:0	7.2	2.2	0.3	0.6
15	18:1(<i>n</i> -9)/20:3(<i>n</i> -9)	1.3	6.7	12.1	1.5
16	16:0/20:3(<i>n</i> -9), 18:0/22:5(<i>n</i> -3)	3.5	5.2	3.6	7.0
17	18:0/20:4(<i>n</i> -6)	2.9	9.2	12.5	3.8
18	15:0/15:0	1.6	0.3	0.5	0.2
19	18:1(<i>n</i> -9)/18:1(<i>n</i> -9)	11.2	7.3	2.2	3.4
20	16:0/18:1(<i>n</i> -9), 18:0/18:2(<i>n</i> -6)	22.4	6.8	3.9	10.0
21	18:0/20:3(<i>n</i> -6)	8.1	2.3	1.0	8.9
22	16:0/16:0	0.3	2.7	1.5	4.5
23	18:0/20:3(<i>n</i> -9)	3.2	20.6	31.0	7.3
24	17:0/18:1(<i>n</i> -9)	0.3	0.5	0.8	0.8
25	16:0/17:0	0.4	0.2	tr	0.6
26	18:1(<i>n</i> -9)/20:1(<i>n</i> -9)	0.4	0.4	tr	0.4
27	16:0/20:1(<i>n</i> -9)	0.6	0.2	1.3	0.3
28	18:0/18:1(<i>n</i> -9)	8.5	8.0	0.6	37.5
29	16:0/18:0	1.8	1.2	1.0	0.4
30	18:0/18:0	0.3	0.4	tr	1.7

hydrolysing the more unsaturated species, resulting in a more limited DAG profile. In particular, these data would support the concept of hormone-sensitive pools of PtdCho which might be selectively found in a particular cell membrane.

All significant increases are observed in polyunsaturated DAG forms with three or more double bonds, most of which are found as major species in PtdIns. In fact, when the PtdIns pattern (Figure 5b) is overlaid on the basal DAG pattern (Figure 2), the resultant profile is very similar to that for DAG obtained from cells stimulated with bombesin for 25 s and, to a lesser extent, 5 min. This strongly suggests that PtdIns or a phosphorylated derivative (i.e. PtdIns_P, which probably has a similar fatty acid profile [16]) is the major source at early time points. The DAG profile at 30 min is elevated in some species that are only minor components of PtdIns suggesting that at least some of the DAG is generated from an alternative phospholipid, probably PtdCho, at this time.

These time-dependent changes in elevated species indicate sequential activation of several phospholipases with different substrate specificities and/or within different cellular compart-

ments. The discovery of multiple forms of phospholipase C in many tissues [29–31] lends support to this idea since it is unlikely that more than one form would be present in a cell if they are all activated identically and/or show identical substrate specificities. The finding that only polyunsaturated species significantly increase after cell stimulation may be partly explained by the fact that these polyunsaturated forms tend to disrupt and loosen the ordered arrangement of lipid bilayers to a greater extent than more saturated species, thus making the former more accessible for phospholipases.

Comparisons of the 1,2-DAG profiles obtained here with those obtained by Divecha et al. [22], also from Swiss 3T3 mouse fibroblasts but using [³H]glycerol labelling, showed that we find less 18:1/18:1 and 18:0/20:4(*n*-6) and no 18:0/22:4. The limited changes we observed after bombesin stimulation were mostly in species whose contribution to the total mass were small (Figure 2). This, together with the fact that they were mostly poorly resolved from other quantitatively more important species under the conditions employed by Divecha et al., probably explains why that group did not find the significant changes we observed.

The 1,2-DAG pattern for 3T3 cells is very similar to that for human SK-N-SH neuroblastoma cells with both showing similar changes in 18:0/20:3(*n*-9), 18:0/20:4(*n*-6) and 18:1(*n*-9)/20:3(*n*-9) when stimulated even though the latter used the muscarinic agonist, carbachol [16], rather than bombesin. This would support a similar mode of action for both these agonists.

Work with rat hepatocytes showed that, although vasopressin stimulated an increase in all species, the largest proportionate increases were in the polyunsaturated species, particularly 16:0/20:4(*n*-6) and 18:0/20:4(*n*-6), the second of which increased steadily with increasing incubation time [14]. This is similar to our findings. Work on IIC9 hamster fibroblasts found that α -thrombin stimulated large, but transient, increases in these species since they returned to basal levels within 5 min whereas other species such as 16:0/20:5(*n*-3) and 18:0/20:5(*n*-3) showed a sustained elevation [9]. Our results show these latter two species were maximal at 30 min bombesin stimulation, suggesting similarities to the later phase of the hamster fibroblast α -thrombin-stimulated response, but differences from the earlier phase.

The very low levels of 1-alkenyl-2-acylglycerol detected in 3T3 fibroblasts support the belief that PtdEtn (where this is a major molecular class) is not hydrolysed after agonist stimulation. The lack of any observable change in the absolute amount of AAG or in its molecular-species pattern at 25 s, 5 min or 30 min is surprising since this suggests that bombesin challenge does not stimulate the hydrolysis of 1-alkyl-2-acyl-PtdCho (the presumed parent phospholipid) by either a phospholipase C or phospholipase D/PtdOH phosphohydrolase pathway. The AAG profile is similar to that from total cellular PtdCho (results not shown) suggesting that a hydrolytic pathway maintains basal levels of this lipid but is not responsive to stimulation with bombesin. Alternatively AAG may be synthesized *de novo* and then subsequently converted into PtdCho by attachment of phosphocholine. This pathway would also explain the profile similarities.

These results are in contrast with work with human neutrophils where both AAG and DAG were generated from radiolabelled PtdCho by the phospholipase D pathway after stimulation with phorbol 12-myristate 13-acetate, 1-oleoyl-2-acetylgllycerol, calcium ionophore A23187 or formylmethioninyl-leucinyphenyl-alanine [32]. Similar experiments with Madin-Darby canine kidney cells using 1-[³H]alkyl-2-lyso-PtdCho also showed DAG

and AAG generation after phorbol 12-myristate 13-acetate stimulation [33]. However, other studies with these cells showed that, whereas DAG generation increased after stimulation with this agonist or dioctanoylglycerol, no changes were observed in AAG levels or species profile even though this lipid represented about 9% of the total PtdCho [34]. This discrepancy between results can be explained if a small rapidly labelled pool of 1-alkyl-2-acyl-PtdCho is susceptible to hydrolysis whereas the larger much more slowly labelled pool is not.

Since other studies have already shown that bombesin stimulates a phospholipase D/PtdOH phosphohydrolase pathway in 3T3 fibroblasts with a DRG response which mirrors but is kinetically downstream of PtdOH generation [6,15,17,18], an explanation for the absence of any change in AAG may be that PtdOH phosphohydrolase is unable to hydrolyse 1-alkyl-2-acyl-PtdOH although it is functional for 1,2-diacyl-PtdOH. Molecular-species analysis of PtdOH should indicate whether this possibility is correct.

The changes in DAG species observed in this study demonstrate not only that there may be differences in either phospholipase or phospholipid substrate with time of stimulation, but also suggest differences in cellular response, i.e. possibly differences in isoform of protein kinase C being activated. Although to date there is limited information concerning the specificity of DAG species in activating individual protein kinase C isoforms, it is an attractive possibility that the distinct changes in the DAG species pattern observed in this study (Figure 3) may relate to a distinct pattern of differential activation of kinase isoforms and thus in protein phosphorylation.

This research was supported by a grant from the Wellcome Trust.

REFERENCES

- Peter-Riesch, B., Fahti, M., Schlegel, W. and Wollheim, C. B. (1988) *J. Clin. Invest.* **81**, 1154–1161
- Wolf, B. A., Easom, R. A., Hughes, J. H., McDaniel, M. L. and Turk, J. (1989) *Biochemistry* **28**, 4291–4301
- Pelech, S. L. and Vance, D. E. (1989) *Trends Biochem. Sci.* **14**, 28–30
- Billah, M. M. and Anthes, J. C. (1990) *J. Biol. Chem.* **265**, 281–291
- Exton, J. H. (1990) *J. Biol. Chem.* **265**, 1–4
- Cook, S. J. and Wakelam, M. J. O. (1991) *Cell. Signall.* **3**, 273–282
- Majerus, P. W., Wilson, D. B., Connolly, T. M., Bross, T. E. and Neufeld, E. J. (1985) *Trends Biochem. Sci.* **10**, 168–171
- Berridge, M. J. (1987) *Biochim. Biophys. Acta* **907**, 33–45
- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
- Kiss, Z. and Anderson, W. B. (1990) *J. Biol. Chem.* **265**, 7345–7350
- Harris, W. E. and Bursten, S. L. (1992) *Biochem. J.* **281**, 675–682
- Pessin, M. S. and Raben, D. M. (1989) *J. Biol. Chem.* **264**, 8729–8738
- Pessin, M. S., Baldassare, J. J. and Raben, D. M. (1990) *J. Biol. Chem.* **265**, 7959–7966
- Augert, G., Bocckino, S. B., Blackmore, P. F. and Exton, J. H. (1989) *J. Biol. Chem.* **264**, 21689–21698
- Plevin, R., Cook, S. J., Palmer, S. and Wakelam, M. J. O. (1991) *Biochem. J.* **279**, 559–565
- Lee, C., Fisher, S. K., Agranoff, B. W. and Hajra, A. K. (1991) *J. Biol. Chem.* **266**, 22837–22846
- Cook, S. and Wakelam, M. J. O. (1989) *Biochem. J.* **263**, 581–587
- Cook, S. J., Briscoe, C. P. and Wakelam, M. J. O. (1991) *Biochem. J.* **280**, 431–438
- Ohanian, J., Ollerenshaw, J., Collins, P. and Heagerty, A. (1990) *J. Biol. Chem.* **265**, 8921–8928
- Chilton, F. H., Cluzel, M. and Triggiani, M. (1990) *Lipids* **26**, 1021–1027
- Nishizuka, Y. (1988) *Nature (London)* **334**, 661–665
- Divecha, N., Lander, D. J., Scott, T. W. and Irvine, R. F. (1991) *Biochim. Biophys. Acta* **1093**, 184–188
- Kennerly, D. A. (1987) *J. Biol. Chem.* **262**, 16305–16313
- Kito, M., Takamura, H., Narita, H. and Urade, R. (1985) *J. Biochem. (Tokyo)* **98**, 327–331
- Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Takamura, H., Kasai, H., Arita, H. and Kito, M. (1990) *J. Lipid Res.* **31**, 709–717
- Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Nidel, J. E. and Bell, R. M. (1986) *J. Biol. Chem.* **261**, 8597–8600
- Paterson, A., Plevin, R. and Wakelam, M. J. O. (1991) *Biochem. J.* **280**, 829–830
- Rhee, S. G., Kim, H., Suh, P.-G. and Choi, W. C. (1991) *Biochem. Soc. Trans.* **19**, 337–341
- Rhee, S. G., Suh, P.-G., Ryu, S. H. and Lee, S. Y. (1989) *Science* **244**, 546–550
- Meldrum, E., Parker, P. J. and Carozzi, A. (1991) *Biochim. Biophys. Acta* **1092**, 49–71
- Agwu, D. E., McPhail, L. C., Chabot, M. C., Daniel, L. W., Wykle, R. L. and McCall, C. E. (1989) *J. Biol. Chem.* **264**, 1405–1413
- Daniel, L. W., Waite, M. and Wykle, R. L. (1986) *J. Biol. Chem.* **261**, 9128–9132
- Robinson, M. and Warne, T. R. (1991) *Biochim. Biophys. Acta* **1085**, 63–70