Muscarinic stimulation of SK-N-BE(2) human neuroblastoma cells elicits phosphoinositide and phosphatidylcholine hydrolysis: relationship to diacylglycerol and phosphatidic acid accumulation

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Muscarinic stimulation of the human neuroblastoma cell line SK-N-BE(2) elicits hydrolysis of phosphoinositides and phosphatidylcholine (PtdCho) and produces a rapid and sustained elevation of diacylglycerol (DG) mass. PtdIns(4,5) P_2 cleavage by phospholipase C (PLC) occurred immediately after carbachol (CCh) addition, and phosphoinositide hydrolysis was then sustained for at least 5 min. Cell stimulation, after extensive PtdCho labelling by long-term [3 H]choline administration, resulted in an enhanced release of [3 H]phosphocholine (PCho) into the external medium; enhanced [3 H]PCho release, which occurred with a 15 s delay with respect to CCh addition, was particularly pronounced within the first minute of stimulation and proved to be caused by PtdCho-specific PLC activation. In fact, when cells were exposed to [3 H]choline for a short period, to extensively label the intracellular PCho pool but not PtdCho, stimulation did not result in an enhanced

release of [3 H]PCho into the medium. PtdCho-specific phospholipase D (PLD) activation was documented by the accumulation of [3 H]phosphatidylethanol in cells prelabelled with [3 H]myristic acid and stimulated in the presence of 1% (v/v) ethanol; this metabolic pathway, however, proved to be a minor one leading to generation of phosphatidic acid (PtdOH) during cell stimulation, whereas DG production by the sequential action of PtdCho-specific PLD and PtdOH phosphohydrolase was not observed. Studies on cells which were double-labelled with [3 H]myristic acid and [14 C]arachidonic acid indicated that within 15 s of stimulation DG is uniquely derived from PtdIns(4,5) P_2 , whereas PtdCho is the major source at later times. Evidence is provided that rapid and selective conversion of phosphoinositide-derived DG into PtdOH may play an important role in determining the temporal accumulation profile of DG from the above-mentioned sources.

INTRODUCTION

It is now well established that hydrolysis of both phosphoinositides and phosphatidylcholine (PtdCho) may account for accumulation of diacylglycerol (DG) during cell stimulation with Ca^{2+} -mobilizing agonists [1–4]. Cleavage of PtdIns(4,5) P_2 by receptor-mediated activation of phospholipase C (PLC) has been invariably regarded as a major source of DG during cell stimulation [1]. PtdCho may provide an additional source of DG through two distinct pathways: first, through hydrolysis by PtdCho-specific PLC; and secondly, via hydrolysis by PtdCho-specific phospholipase D (PLD) to choline and phosphatidic acid (PtdOH), followed by cleavage of the latter metabolite to phosphate and DG by PtdOH phosphohydrolase [2–4].

Stimulation of phosphoinositide hydrolysis elicited by muscarinic agonists is a well-established phenomenon in a number of human neuroblastoma cell lines [5–8]; fewer studies have, conversely, dealt with muscarinic-receptor-mediated PtdCho breakdown. In particular, the relative contribution made by phosphoinositide and PtdCho hydrolysis to DG accumulation, and the metabolic routes through which PtdCho provides DG, remain largely to be investigated. In a recent study, Lee and coworkers analysed DG and PtdOH molecular species produced upon muscarinic stimulation of SK-N-SH human neuroblastoma [9]. These authors demonstrated that shortly after stimulation DG, mainly originates from phosphoinositides, whereas at later times PtdCho is a major source of this lipid through PLC

hydrolysis; furthermore it was reported that SK-N-SH cells lack PtdCho-specific PLD activity [9]. PtdCho-specific PLD activation following muscarinic stimulation has, conversely, been demonstrated in LAN-2 cells [8]; however, the issue of whether PtdOH originating from PtdCho hydrolysis undergoes dephosphorylation by PtdOH phosphohydrolase, thus providing a source of DG, has not been investigated. In this paper we report a study aimed at investigating the sources of DG as well as the metabolic routes by which DG is produced during muscarinic stimulation of the human neuroblastoma cell line SK-N-BE(2).

EXPERIMENTAL

Materials

[1- 14 C]Arachidonic acid (54 mCi/mmol), [9,10-(n)- 3 H]myristic acid (53 Ci/mmol), *myo*-[2- 3 H]inositol (17.5 Ci/mmol), [*methyl*- 3 H]choline chloride (75 Ci/mmol) and adenosine 5'-[γ - 32 P]-triphosphate (3 Ci/mmol) were from Amersham Corp., Amersham, Bucks., U.K. Carbachol (CCh) and choline chloride were from Fluka, Buchs, Switzerland. Dowex-1 chloride (8% cross-linked; 200–400 mesh), Hepes, fatty-acid-free BSA and 1-octadecanoyl-2-(5,8,11,14-icosatetraenoyl)glycerol were from Sigma, St. Louis, MO, U.S.A. *Escherichia coli* DG kinase was from Lipidex, Westfield, NJ, U.S.A. R59022 was from Janssen Chimica, Belgium, Silica gel 60 t.l.c. and high-performance t.l.c.

Abbreviations used: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PCho, phosphocholine; AA, arachidonic acid; MA, myristic acid; DG, 1,2-diacylglycerol; MG, monoacylglycerol; TG, triacylglycerol; PtdOH, phosphatidic acid; PtdEtOH, phosphatidylethanol; PLC, phospholipase C; PLD, phospholipase D; CCh, carbachol.

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plates were from Merck, Darmstadt, Germany. MEM, RPMI 1640 and fetal calf serum were from Flow Laboratories, U.K.

Cell culture

The SK-N-BE(2) human neuroblastoma cell line was obtained through the courtesy of Dr. Lawrence Helson (Memorial Sloane-Kettering Hospital, New York, NY, U.S.A.). Cells were grown at 37 °C in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 i.u./ml penicillin and 100 µg/ml streptomycin, in a humidified atmosphere with 5% (v/v) CO₂.

Cell labelling with [³H]Ins, extraction and analysis of [³H]InsPs and [³H]phosphoinositides

Cells grown in 25 cm² flasks (9 × 10⁴ cells/cm²) were labelled for 24 h with 12.5 µCi of [³H]Ins in 2.5 ml of MEM supplemented with 10% (v/v) fetal calf serum. Cells were then washed with RPMI-1640 medium, incubated for an additional hour in label-free RPMI-1640 medium containing 10 mM Li⁺ and stimulated by addition of 1 mM CCh directly into the medium. The reaction was terminated by rapid aspiration of the medium and addition of 3.0 ml of cold 10% (w/v) trichloroacetic acid; cells were then scraped off, transferred into test tubes and, after standing for 30 min on ice, were centrifuged; the pellet was washed twice with 1 ml of 5% (w/v) trichloroacetic acid containing 1 mM EGTA and the washings were mixed with the supernatant. The acid-soluble extract was washed three times with 3 vol. of water-saturated diethyl ether, neutralized with NaHCO₃ and individual classes of [³H]InsPs were then separated by anion-exchange chromatography on Dowex-1 (formate form) as described [10]. For quantitation of individual [³H]phosphoinositide classes, the washed trichloroacetic acid precipitate was extracted twice with 1.5 ml of chloroform/methanol/12 M HCl (100:100:1) and once with 1.5 ml of chloroform/methanol/12 M HCl (200:100:1); the three extracts were combined and 1.5 ml of chloroform and 1.1 ml of 0.1 M HCl were added to separate the phases. [³H]-Phosphoinositides were de-acylated and glycerophospho-derivatives separated by ion-exchange chromatography by the method of Creba et al. [11].

Mass determination of Ins(1,4,5)P₃ and DG

Cells grown to confluency in 35 mm-diam. plates (10⁶ cells/plate) were incubated for 1 h in RPMI 1640 medium and stimulated by rapid shifting to 1.0 ml of Hepes-buffered saline (137 mM NaCl, 27 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5.6 mM D-glucose, 20 mM Hepes, pH 7.4) containing 1 mM CCh. The reaction was terminated by addition of 1.0 ml of cold 20% (w/v) trichloroacetic acid; cells were then scraped off, transferred into test tubes and, after standing for 30 min on ice, were centrifuged. The acid-soluble extract was washed three times with 3 vol. of water-saturated diethyl ether neutralized with NaHCO₃ and Ins(1,4,5)P₃ mass was measured by a commercially available specific radioreceptor assay (Amersham Corp., Amersham, Bucks., U.K.).

For determination of DG mass cells were grown and stimulated as described above and the reaction was terminated by addition of 1 ml of ice-cold methanol. Cells were then scraped off and lipid extraction performed according to the method of Bligh and Dyer [12]. DG mass was determined by conversion of DG into [³²P]PtdOH, after reaction with [γ -³²P]ATP in the presence of DG kinase from *E. coli*, essentially as described by [13]. Calibration

was performed using 1-octadecanoyl-2-(5,8,11,14-icosatetraenoyl) glycerol.

Cell labelling with [³H]choline and analysis of water-soluble choline metabolites

Cells grown in 35 mm-diam. plates were labelled for 16 h with 1 µCi of [³H]choline added to the culture medium and were then allowed to grow for an additional 24 h in label-free medium. Before stimulation, cells were incubated at 37 °C for 1 h in serum-free RPMI 1640 medium containing 1 mM of label-free choline chloride and were washed three times with Hepes-buffered saline. Stimulation was initiated by rapidly shifting the cells into 1 ml of Hepes-buffered saline containing 1 mM CCh, allowed to proceed for the required time at 37 °C and terminated by rapid aspiration of the incubation medium and addition of ice-cold methanol to cells. For each incubation period a control was carried out in which cells were shifted to Hepes-buffered saline free of CCh. Before analysis, the aspirated medium was centrifuged at 900 *g* for 3 min. Intracellular water-soluble metabolites were separated from phospholipids by extraction according to Bligh and Dyer [12]. Water-soluble [³H]choline metabolites were separated by t.l.c. on silica-gel 60 plates as described by Yavin [14], or by extraction with tetraphenylboron in heptanone as described [15].

Cell labelling with [¹⁴C]arachidonic acid (AA) and [³H]myristic acid (MA), lipid extraction and separation

Cells grown in 35 mm diam. plates were incubated for 4 h with 2 ml of RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, to which 0.25 µCi of [¹⁴C]AA and/or 0.65 µCi of [³H]MA had been added previously. Labelling was terminated by aspirating the medium and washing cells twice with Hepes-buffered saline containing 0.1% defatted BSA; cells were then re-incubated in label- and serum-free RPMI 1640 medium for 1 h. For cell stimulation the medium was replaced rapidly by 1 ml of Hepes-buffered saline containing 1 mM CCh; the reactions was stopped by an equal volume of cold 20% (w/v) trichloroacetic acid. Cells were then scraped off, transferred to test tubes and centrifuged; lipids were extracted from the resulting pellet according to [12]. Two-dimensional separation of phospholipids was carried out on high-performance t.l.c. plates as described [16]. Neutral lipids and free fatty acids were separated on high-performance t.l.c. plates by running samples in the upper phase of ethyl acetate/iso-octane/acetic acid/water (110:50:20:100) to half-plate height and, after drying under nitrogen, in the same direction to full-plate height in hexane/diethyl ether/methanol/acetic acid (90:20:3:2). PtdOH and phosphatidylethanol (PtdEtOH) were separated by sample development on high-performance t.l.c. plates using the upper phase of ethyl acetate/iso-octane/acetic acid/water (130:20:30:100). PtdEtOH standard was prepared by egg-yolk PtdCho transphosphatidylation with cabbage PLD as described [17]. Lipid spots were visualized under iodine and scraped from the plates into counting vials; radioactivity was measured with a Packard Tricarb 1500 scintillation counter, using Optifluor (Packard) as the scintillation fluid. Protein determination was carried out using BSA as a standard [18].

RESULTS

CCh stimulates hydrolysis of phosphoinositides and PtdCho by PLC and elicits DG accumulation

Figure 1 shows that muscarinic stimulation elicits phosphoinositide hydrolysis in SK-N-BE(2) human neuroblastoma cells.

Figure 1 Time course of CCh-elicited labelling changes in total [^3H]InsPs and [^3H]phosphoinositides

Cells were labelled for 24 h with [^3H]Ins and stimulated for the indicated periods with 1 mM CCh in the presence of 10 mM Li^+ ; [^3H]InsPs ($\square$) (upper panel), [^3H]PtdIns ($\blacksquare$) (middle panel), [^3H]PtdIns(4)P ($\circ$) and [^3H]PtdIns(4,5) P_2 ($\bullet$) (lower panel) were then extracted and separated as described in the Experimental section. Each point is the mean $\pm$ S.D. of results from four different experiments.

The accumulation time course of [^3H]InsPs, as assessed during stimulation with 1 mM CCh in the presence of 10 mM Li^+ of cells prelabelled with [^3H]Ins, is shown in Figure 1 (upper panel). Figure 1 also shows the time course of labelling changes occurring in PtdIns (middle panel) as well as in PtdIns(4)P and PtdIns(4,5) P_2 (lower panel). Results clearly demonstrate that PtdIns(4,5) P_2 cleavage occurred immediately after agonist addition and phosphoinositide hydrolysis was then sustained for at least 5 min. Maximal accumulation of the Ca^{2+} -mobilizing isomer, Ins(1,4,5) P_3 , as monitored by means of a specific radioreceptor binding assay, was observed 10–15 s after cell stimulation (Figure 2). The question as to whether PtdIns and PtdIns(4)P are directly

Figure 2 Time course of Ins P_3 accumulation in CCh-stimulated cells

Cells were stimulated with 1 mM CCh for the indicated periods as described and Ins(1,4,5) P_3 determination was performed by a specific radio receptor assay. Each point is the mean $\pm$ S.D. of results from four different experiments. Statistical significance: * $P < 0.01$, versus controls, after paired data analysis.

hydrolysed during prolonged cell stimulation, or whether their reduction of labelling is in connection with the replenishment of the PtdIns(4,5) P_2 pool was disregarded: therefore, we generically refer to 'phosphoinositide hydrolysis' whenever necessary. It must be pointed out that the pronounced decrease in radioactivity associated with PtdIns (Figure 1, lower panel) does not reflect a net loss of phospholipid mass. PtdIns mass, as assessed by phosphate analysis according to [19], was 11.4 ± 0.8 nmoles/mg of protein in unstimulated cells and after 5 min of stimulation it altered to 10.5 ± 0.7 nmoles/mg of protein (results are means $\pm$ S.D., $n = 4$, not significant by Student's t test). A plausible explanation for the phenomenon is that PtdIns is actively resynthesized during stimulation by utilization of unlabelled Ins from the medium.

To investigate whether PtdCho undergoes hydrolysis during cell muscarinic stimulation, [^3H]choline was used to label PtdCho; changes in the radioactivity content of PtdCho and of water-soluble metabolites resulting from PLC and PLD hydrolysis, i.e. phosphocholine (PCho) and choline respectively, were then monitored at various times after agonist addition (both in the intra- and extra-cellular media) and referred to controls.

After long-term labelling with $1 \mu\text{Ci}$ of [^3H]choline, PtdCho incorporated $153 (\pm 23) \times 10^3$ d.p.m./mg of protein (more than 95% of the total phospholipid radioactivity) whereas radioactivity found in intracellular PCho and free choline was $40.0 (\pm 8.4) \times 10^3$ and $2.7 (\pm 0.4) \times 10^3$ d.p.m./mg of protein respectively (values are means $\pm$ S.D. of four different determinations). Shifting of cells to a fresh medium led to release of water-soluble labelled choline metabolites into the extracellular compartment, even in the absence of stimulation; after 5 min of re-incubation this averaged $1.8 (\pm 0.3) \times 10^3$ d.p.m./mg of protein for [^3H]PCho and $0.7 (\pm 0.3) \times 10^3$ d.p.m./mg of protein for [^3H]choline (means $\pm$ S.D., $n = 6$ in both cases). In Figure 3 it can be seen that cell stimulation with 1 mM CCh enhanced the release of [^3H]PCho, but not of [^3H]choline, into the external medium, thus indicating that muscarinic stimulation elicits PtdCho hydrolysis by PLC activation. The phenomenon was particularly pronounced between 15 and 60 s, at which time [^3H]PCho release

Figure 3 Time course of CCh-induced release of [^3H]choline and [^3H]PCho into the extracellular medium

[^3H]Choline-labelled cells were exposed to 1 mM CCh or control buffer for the indicated times, after which the extracellular medium was aspirated and analysed for [^3H]choline and [^3H]PCho content as described in the Experimental section. Each point is the mean $\pm$ S.D. of results from six different experiments. Statistical significance $^*P < 0.01$, versus controls, after paired data analysis.

Table 1 Failure of CCh to enhance [^3H]PCho release into the medium after short-term labelling of cells with [^3H]choline

Cells were labelled with 5 μCi of [^3H]choline for 15 min and re-incubated for 5 min in fresh label-free medium in the presence or absence of 1 mM CCh. At the end of the reaction the medium was aspirated, intracellular water-soluble metabolites and lipids were separated and analysed as described in the Experimental section. Results are means $\pm$ S.D. of four independent experiments.

	10 ⁻³ $\times$ Incorporation of label (d.p.m./mg of protein)	
	Unstimulated cells	Stimulated cells
[^3H]PtdCho	5.4 $\pm$ 0.7	5.3 $\pm$ 0.6
[^3H]PCho (intracellular)	193.2 $\pm$ 14.7	189.1 $\pm$ 15.6
[^3H]PCho (extracellular)	45.7 $\pm$ 4.2	44.1 $\pm$ 3.3

was approx. 80 % of that observed at 5 min. Cell stimulation did not significantly affect labelling of PtdCho or of water-soluble intracellular metabolites (results not shown). Enhanced release of [^3H]PCho into the external medium was not caused by stimulated release by CCh of the intracellular [^3H]PCho pool. Table 1 shows that, after short-term exposure of cells to [^3H]choline, the intracellular pool of PCho was extensively labelled, whereas a poor incorporation of radioactivity into PtdCho occurred. In these conditions enhanced release of [^3H]PCho into the medium by CCh stimulation did not occur.

The effect brought about by muscarinic stimulation on cell DG mass was investigated. A basal value of 1.4 ± 0.2 nmoles/mg of protein was detected for this lipid, comparable with that reported recently for another human neuroblastoma cell line [9]. Figure 4 shows that CCh addition elicited a rapid and continuous accumulation of DG, leading, after 5 min, to a 50 % increase over the basal level.

Table 2 Labelling of major cell phospholipids by [^{14}C]AA and [^3H]MA

Cells were labelled for 4 h with 0.25 μCi of [^{14}C]AA and 0.65 μCi of [^3H]MA, lipids were extracted and the phospholipids analysed as described in the Experimental section. Results are means $\pm$ S.D. of four independent determinations. Abbreviation: PtdSer, phosphatidylserine.

	10 ⁻³ $\times$ Incorporation of label (d.p.m./mg of protein)		
Phospholipid	[^{14}C]AA	[^3H]MA	$^{14}\text{C}/^3\text{H}$
PtdCho	74.8 $\pm$ 6.0	391.4 $\pm$ 9.2	0.19
PtdEtn	134.2 $\pm$ 7.8	43.3 $\pm$ 3.0	3.10
PtdIns	71.3 $\pm$ 6.7	16.8 $\pm$ 1.3	4.24
PtdSer	15.2 $\pm$ 2.6	8.1 $\pm$ 0.3	1.88

Figure 4 Time course of DG accumulation in CCh-stimulated cells

Cells were stimulated with 1 mM CCh for the indicated periods as described and DG determination performed as described in the Experimental section. Basal DG (1.4 ± 0.2 nmoles/mg of protein) was subtracted from values of CCh-stimulated samples. Each point is the mean $\pm$ S.D. of results from four different experiments. Statistical significance: $^*P < 0.01$, versus controls, after paired data analysis.

Accumulation of radioactive metabolites during stimulation of cells double-labelled with [^{14}C]AA and [^3H]MA

The temporal accumulation profile of lipid metabolites deriving from phosphoinositide and PtdCho hydrolysis was investigated following stimulation of cells double-labelled with [^3H]MA and [^{14}C]AA.

In the labelling conditions employed the $^{14}\text{C}/^3\text{H}$ isotopic ratio in PtdIns was 20-fold higher than that observed in PtdCho (Table 2); the isotopic ratios are expected to be maintained in the metabolites of the two phospholipids, provided that no process of deacylation–reacylation occurs. Figure 5 (upper panel) shows that at the earliest time-points tested (i.e. 10 and 15 s) a significant increase in accumulation of ^{14}C but not of ^3H took place, suggesting that shortly after stimulation only PtdIns(4,5) P_2 provides DG. At later time-points, ^3H accumulation was dramatically increased, well exceeding that observed for ^{14}C ; by 30 s of stimulation the $^{14}\text{C}/^3\text{H}$ isotopic ratio changed to about 0.56 and held at a level between this value and 0.37 throughout the remainder of the stimulation time course, thus pointing to a substantial contribution of PtdCho in DG accumulation. In our

Figure 5 CCh-elicited accumulation of radioactive DG (upper panel) and PtdOH (lower panel) in cells double-labelled with [^{14}C]AA and [^3H]MA

Cells were labelled with [^{14}C]AA and [^3H]MA and stimulated with 1 mM CCh; at the indicated times the reaction was stopped, lipids extracted and DG and PtdOH separated as described in the Experimental section. Control values ($\times 10^{-3}$ d.p.m.) (2.6 ± 0.2 for [^{14}C]DG, 11.4 ± 0.3 for [^3H]DG, 0.5 ± 0.1 for [^{14}C]PtdOH and 2.3 ± 0.2 for [^3H]PtdOH) were subtracted from values of CCh-stimulated samples. Each point is the mean $\pm$ S.D. of results from four different experiments. Statistical significance: $*P < 0.01$, versus controls, after paired data analysis.

system, triacylglycerol (TG) incorporated [^{14}C]AA and [^3H]MA with a $^{14}\text{C}/^3\text{H}$ ratio (0.16) close to that observed for PtdCho: the possibility that TG could provide an additional source of DG by deacylation had thus to be considered. If this were the case, however, release of labelled free fatty acids would also have been expected. Results reported in Figure 6 (lower panel), conversely, demonstrate that neither [^{14}C]AA nor [^3H]MA was released to any significant extent throughout the stimulation time course; furthermore, when cells were stimulated after labelling with [^{14}C]cis-9-octadecenoic acid, i.e. the TG major fatty-acid constituent, radioactivity accumulation took place in DG, but no increase in labelling was observed in free fatty acids (results not shown). Finally, we could not detect any change of radioactivity in TG during cell stimulation. These results, albeit circumstantial, point to TG as an unlikely source of DG. It should also be noted

Figure 6 CCh-elicited accumulation of radioactive MG (upper panel) and free fatty acids (lower panel) in cells double-labelled with [^{14}C]AA and [^3H]MA

Cells were labelled with [^{14}C]AA and [^3H]MA and stimulated with 1 mM CCh; at the indicated times the reaction was stopped, lipids extracted and MG and free fatty acids separated as described in the Experimental section. Control values ($\times 10^{-3}$ d.p.m.) of 0.6 ± 0.1 for [^{14}C]MG, 3.2 ± 0.1 for [^3H]MG, 2.7 ± 0.2 for [^{14}C]AA and 6.8 ± 0.3 for [^3H]MA were subtracted from values of CCh-stimulated samples. Each point is the mean $\pm$ S.D. of results from four different experiments. Statistical significance: $*P < 0.01$, versus controls, after paired data analysis.

that the $^{14}\text{C}/^3\text{H}$ ratio observed in late accumulation of DG, as compared with that observed in phosphatidylethanolamine (PtdEtn), suggests that during this period this major phospholipid class does not provide a source of DG.

Changes in DG labelling were accompanied by radioactivity accumulation in PtdOH (Figure 5, lower panel), the high $^{14}\text{C}/^3\text{H}$ ratio observed throughout the stimulation time course being strongly suggestive that PtdOH is mainly produced through phosphorylation of phosphoinositide-derived DG. To test this hypothesis a set of studies were performed with the compound R59022. R59022 is regarded as a specific inhibitor of DG kinase [20]; however, it has also been shown to inhibit agonist binding at the level of muscarinic receptors by a competitive mechanism [21]. Table 3 shows the effect of 10 μM R59022 on accumulation of radioactivity into DG and PtdOH, following stimulation of cells prelabelled with [^{14}C]AA: it can be seen that R59022 reduced the increase of [^{14}C]PtdOH by more than 80% but no corresponding increase in [^{14}C]DG levels was observed. These results can be explained on the basis of the combined effects of R59022 at the level of DG kinase and muscarinic receptors.

Deacylation proved to be a minor route for DG removal during stimulation. Figure 6 shows that a small and transient accumulation of [^{14}C]monoacylglycerol (MG) took place shortly

Table 3 Effects of 10 μ M R59022 on [14 C]DG and [14 C]PtdOH accumulation after stimulation of cells prelabelled with [14 C]AA

Cells prelabelled for 4 h with 0.25 μ Ci of [14 C]AA were pre-incubated for 15 min in Hepes-buffered saline in the presence and absence of 10 μ M R59022; 1 mM CCh was then added to the medium and the reaction allowed to proceed for 5 min. After this time, the reaction was stopped, lipids were extracted and DG and PtdOH separated as described in the Experimental section. Cell pre-incubation with 10 μ M R59022 did not affect basal levels of [14 C]DG and [14 C]PtdOH. Each value is the mean $\pm$ S.D. of results from four different experiments. Statistical significance: * P < 0.01, as compared with cells stimulated in the absence of R59022, after Student's t test.

Incubation conditions	10 ⁻³ $\times$ Accumulation of label (d.p.m./mg of protein)	
	[14 C]DG	[14 C]PtdOH
Basal	2.7 $\pm$ 0.3	0.4 $\pm$ 0.1
1 mM CCh	3.9 $\pm$ 0.4	6.0 $\pm$ 0.5
1 mM CCh + 10 μ M R59022	3.7 $\pm$ 0.4	1.5 $\pm$ 0.2*

Table 4 Effects of 1% (v/v) ethanol on CCh-elicited accumulation of [3 H]DG and [3 H]PtdOH in cells prelabelled with [3 H]MA

Cells prelabelled for 4 h with 0.65 μ Ci of [3 H]MA were pre-incubated for 15 min in Hepes-buffered saline in the presence or absence of 1% (v/v) ethanol; 1 mM CCh was then added to the medium and the reaction allowed to proceed for 5 min. After this time the reaction was stopped, lipids extracted and DG and PtdOH separated as described in the Experimental section. Cell pre-incubation with 1% (v/v) ethanol did not affect basal levels of [3 H]DG and [3 H]PtdOH. Each value is the mean $\pm$ S.D. of results from four different experiments. Statistical significance: * P < 0.05, as compared with cells stimulated in the absence of ethanol after Student's t test.

Incubation conditions	10 ⁻³ $\times$ Accumulation of label (d.p.m./mg of protein)	
	[3 H]DG	[3 H]PtdOH
Basal	11.2 $\pm$ 0.5	2.2 $\pm$ 0.2
1 mM CCh	13.8 $\pm$ 0.6	4.4 $\pm$ 0.3
1 mM CCh + 1% (v/v) ethanol	13.6 $\pm$ 0.6	3.9 $\pm$ 0.2*

Figure 7 CCh-elicited accumulation of radioactive PtdEtOH in cells double-labelled with [14 C]AA and [3 H]MA

Cells were labelled with [14 C]AA and [3 H]MA as described and incubated for 15 min in Hepes-buffered saline containing 1% (v/v) ethanol before stimulation with 1 mM CCh. At the indicated times the reaction was stopped, lipids extracted and PtdEtOH separated as described in the Experimental section. Control values of d.p.m. (180 $\pm$ 60 for [3 H]PtdEtOH and 85 $\pm$ 30 for [14 C]PtdEtOH) were subtracted from values of CCh-stimulated samples. Each point is the mean $\pm$ S.D. of results from seven different experiments. Statistical significance: * P < 0.01, versus controls, after paired data analysis.

after cell stimulation, but was not accompanied by intracellular (as well as extracellular; results not shown) elevation of free [14 C]AA. Accumulation of neither [3 H]MG nor of free [3 H]MA was observed.

Cell stimulation elicits activation of a PtdCho-specific PLD, as detected by accumulation of [3 H]MA-labelled PtdEtOH

Experiments reported in Figure 3 had demonstrated that release of [3 H]choline into the medium was not significantly enhanced during cell stimulation, a higher release on average only being observed in comparison with controls. To further study whether

any PLD was involved in PtdOH production, cells labelled as shown in Table 2 were stimulated with 1 mM CCh in the presence of 1% (v/v) ethanol and accumulation of [3 H]PtdEtOH and [14 C]PtdEtOH were measured at various time intervals. Figure 7 shows that cell stimulation in the presence of ethanol led to a significant accumulation of radioactivity in [3 H]PtdEtOH, mainly occurring between 15 and 60 s, whereas no increase of [14 C]PtdEtOH was observed. These results indicate that muscarinic cell stimulation elicits hydrolysis by PLD of PtdCho but not of phosphoinositides (and, indeed, of PtdEtn). To assess whether DG production occurred to any extent through the PLD/PtdOH phosphohydrolase pathway, the effect of ethanol on DG accumulation was also investigated. Table 4 shows that stimulation of cells prelabelled with [3 H]MA in the presence of ethanol resulted in an approx. 20% reduction of [3 H]PtdOH accumulation in comparison with cells stimulated in the absence of ethanol, without any effect on DG labelling. Further experiments indicated that neither accumulation of radioactivity in DG and PtdOH, as assessed in cells prelabelled with [14 C]AA, nor the increase of DG mass was affected by the presence of 1% (v/v) ethanol during cell stimulation (results not shown).

DISCUSSION

A major conclusion that can be drawn from this study is that elevation of DG levels during muscarinic stimulation of the human neuroblastoma cell line SK-N-BE(2) originates from hydrolysis of phosphoinositides and PtdCho due to activation of specific PLCs. The metabolic route leading to DG accumulation through the sequential action of a PtdCho-specific PLD and a PtdOH phosphohydrolase does not appreciably operate in our system. Evidence that PtdCho is hydrolysed by a PLC is provided by the enhanced accumulation of [3 H]PCho in the external medium during stimulation of cells prelabelled with [3 H]choline: this proved to be a phenomenon caused by PtdCho hydrolysis and not by an enhanced release into the medium of [3 H]PCho from the intracellular pool. In fact, it was not observed when [3 H]choline was administered to cells for a short period to extensively label the intracellular pool of PCho with poor PtdCho labelling. It should be noted that, according to observations carried out on other cell systems [15,22], PtdCho hydrolysis was fairly transient occurring to a large extent, in our system, within the first minute of stimulation. Experiments carried out in the presence of ethanol indicated that PtdCho hydrolysis by PLD is a minor route leading to PtdOH production, and that DG is not

produced appreciably through PtdOH dephosphorylation. In the presence of ethanol, PLD catalyses a transphosphatidylolation reaction, leading to the production of PtdEtOH at the expense of PtdOH [17]; in these conditions, if PtdOH provides a source of DG through dephosphorylation, decreased DG accumulation would also be expected. In this connection we observed that although stimulation of cells prelabelled with [^3H]MA and [^{14}C]AA in the presence of 1% (v/v) ethanol led to accumulation of [^3H]PtdEtOH (but not of [^{14}C]PtdEtOH), accumulation of radioactivity in [^3H]PtdOH was only marginally affected. In addition, neither elevation of [^3H]DG levels nor of DG mass was affected during cell stimulation in the presence of ethanol.

Experiments on cells double-labelled with [^3H]MA and [^{14}C]AA, indicate that phosphoinositides and PtdCho provide a major source of DG in a temporally sequential fashion: within 15 s of cell stimulation, DG is uniquely derived from PtdIns(4,5) P_2 , whereas shortly after this time DG accumulation is mainly sustained by PtdCho hydrolysis. While the former circumstance is easily explained by the observation that PtdCho hydrolysis occurs with a 15 s delay from cell stimulation, an explanation for the evidence that PtdCho provides by far the largest source of DG at later times, although phosphoinositide cleavage is sustained throughout the stimulation time course, may be provided by the differential rate by which DG from the above-mentioned sources is removed. The high $^{14}\text{C}/^3\text{H}$ isotopic ratio observed in accumulating PtdOH throughout the stimulation time course indicates that phosphoinositide-derived DG is rapidly and selectively converted into PtdOH, by phosphorylation by DG kinase. This view is consistent with the observations that the [^{14}C]PtdOH increase is due to neither [^{14}C]AA incorporation into *de novo* synthesized PtdOH, owing to the constancy of free [^{14}C]AA throughout the stimulation time course, nor is accounted for by phosphoinositide-specific PLD activation, as suggested from the evidence that accumulation of [^{14}C]PtdEtOH after cell stimulation in 1% (v/v) ethanol does not occur in the labelling conditions employed. Circumstantial evidence also came from studies with the putative DG kinase inhibitor R59022.

The evidence that PtdCho-derived DG is poorly, if to any extent, metabolized to PtdOH, is in agreement with recent reports on various cell systems [9,23,24] and the possibility that it may be related to DG kinase specificity for AA-containing DG [25] remains to be investigated. Further efforts, of course, are

required to elucidate the mechanisms leading to PtdCho-specific PLC activation and the role of PtdCho-derived DG.

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