Phorbol ester-sensitive phospholipase D is mainly localized in the endoplasmic reticulum of BHK cells

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The localization of phorbol ester-sensitive phospholipase D (PLD) in baby hamster kidney cells has been investigated by determining the subcellular distribution of the phosphatidylbutanol produced when the cells are incubated with phorbol 12-myristate 13-acetate and n-butanol. Results derived by isolation

of plasma membrane vesicles from intact cells or by subcellular fractionation on a sucrose density gradient suggest the PLD is specific for phosphatidylcholine and its primary site of action is not the plasma membrane but the endoplasmic reticulum.

INTRODUCTION

In many cell types, response to an agonist involves the activation of a phospholipase D (PLD) that specifically degrades phosphatidylcholine (PtdCho) [1–3]. The precise role of PLD in cellular activation is still unclear although there are strong indications that it might be involved in the regulation of mitosis [4,5] and other membrane trafficking events [5–7]. To determine the role of a particular PLD activity it is important to define its subcellular localization. In principle this problem can be approached by fractionating cells and then determining PLD activity in individual fractions. Such a procedure has some disadvantages in that it might not be easy to establish the correct conditions for optimal assay *in vitro*, a number of different isoenzymes might show activity and there is no certainty that stimulated cells will retain enhanced levels of PLD activity after fractionation. PLD activity can be followed in cell fractions by the measurement of either the release of choline or the accumulation of phosphatidate, but in the latter case, because of the rapid further metabolism of phosphatidate, it is usually more practicable to measure accumulation of the non-metabolized transphosphatidyl derivatives of phosphatidate when cells are incubated with agonists in the presence of appropriate alcohols [1–3].

An alternative approach to the localization of PLD activity is to stimulate the enzyme in intact cells in the presence of alcohols and then to measure the phosphatidylalcohol products of reaction in cell subfractions. There has been a recent attempt to apply this method to the activation of PLD by phorbol ester in HeLa cells and by bradykinin in 3T3 Li cells [8]. This procedure is, of course, only useful if the phosphatidylalcohol products stay in the membrane where they are generated before and after cell fractionation; that would be a dubious proposition if they are subject to degradation or to rapid redistribution between other cell membranes by non-specific lipid transfer proteins [9].

We decided to investigate the subcellular site of PLD activation by phorbol 12-myristate 13-acetate (PMA) in baby hamster kidney (BHK) cells because we have developed well-defined procedures for isolation of plasma membrane vesicles from these cells [10,11] and for cell subfractionation on a sucrose density gradient (M. J. Miro Obradors and D. Allan, unpublished work).

An additional reason is that there is considerable background knowledge about the distribution of lipids between the various organelles of these cells [12]. In BHK cells, a PMA-sensitive PLD has previously been described [13] and an endogenous PLD activity seems to be involved in cell proliferative events [5].

Our initial approach was to make use of previous work that has shown that it is possible to isolate plasma membrane vesicles from BHK or HL60 cells by a fairly rapid process involving treatment with merocyanine 540 and iodoacetamide [10,14]. Whatever the intracellular site of action of PLD, if the phosphatidylalcohol product were to be found distributed evenly between plasma membrane vesicles and the membranes of remnant cells, then subcellular fractionation could not be profitable as a means of identifying the site of PLD activation. In contrast, if the concentration of phosphatidylalcohol were higher in the plasma membrane vesicles, it would be likely that the plasma membrane was the site of PLD activation; if it were higher in the remnant cells, then subcellular fractionation might be able to reveal the nature of this intracellular site of PLD activity.

MATERIALS AND METHODS

Cell culture

BHK cells were cultured in minimum essential medium (Glasgow modification; GMEM; Gibco) and labelled to equilibrium with 2 ml of GMEM in the presence of 5% (v/v) foetal bovine serum and either [³H]choline or [³H]acetate as described previously [15,16]. It was assumed that the distribution of radioactivity reflected the relative masses of choline or carbon respectively in each lipid pool.

Measurement of the distribution of choline radioactivity in BHK cells

Cells grown almost to confluence in 3.5 cm plastic dishes in the presence of 1 μ Ci/ml [³H]choline for 48 h were washed three times with 2 ml of serum-free GMEM and then incubated in 1 ml of the same medium containing 100 nM PMA (Sigma Chemical Co, Poole, Dorset, U.K.) (1 μ l of a 0.1 mM solution in DMSO

Abbreviations used: BHK, baby hamster kidney; ER, endoplasmic reticulum; GMEM, Glasgow modification of minimum essential medium; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PtdCho, phosphatidylcholine.

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added to 1 ml of medium) for up to 90 min or with various concentrations of PMA for 20 min. At the end of these incubations, the supernatant medium was carefully removed for measurement of choline metabolites, and lipids were extracted from the cells by the addition of 1.9 ml of methanol/chloroform (2:1, v/v) to the dishes. After transfer of the extracts to glass test tubes, the organic and aqueous phases were split in accordance with the procedure of Bligh and Dyer [17], the chloroform phase being dried on a water pump and lipids being separated by TLC on silica gel 60 plates (Merck, Darmstadt, Germany) in a solvent consisting of chloroform/methanol/acetic acid/water (75:45:12:2, by vol.). After excision of the spots and addition of 0.2 ml of methanol/water/acetic acid (5:3:2, by vol.) followed by 2 ml of Ultima Gold scintillation fluid, the radioactivity in PtdCho and sphingomyelin was measured in a Canberra Packard TC2500 scintillation counter. Samples of the medium from the incubations and the upper phase (cell aqueous fraction) of the Bligh and Dyer phase separation were counted similarly. Choline and phosphocholine in these aqueous samples were separated by descending paper chromatography in 90% (v/v) ethanol, identified by reaction of internal standards with iodine and ferric sulphosalicylate respectively and counted by liquid scintillation as described previously [15].

Measurement of lipid changes caused by PMA in BHK cells and plasma membrane vesicles

Cells grown as above but with 10 μ Ci/ml [3 H]acetate instead of [3 H]choline were incubated for up to 90 min at 37 °C with or without 100 nM PMA in the presence of 1% (v/v) ethanol or 0.2% (v/v) n-butanol. Lipids were extracted from cells as above and were separated on silica gel 60 plates with chloroform/methanol/acetic acid (75:45:3, by vol.). This solvent separated all the major phospholipids, cholesterol and the phosphatidylalcohols, as shown in Figure 1. Phosphatidic acid ran slightly faster than phosphatidylethanolamine, from which it was occasionally not resolved completely. In further experiments, [3 H]acetate-labelled cells were incubated with or without PMA in the presence of 0.2% (v/v) n-butanol and then were induced to release plasma membrane vesicles by incubation with iodoacetamide and merocyanine 540 as described by Whatmore et al. [10,14]. Radioactive spots were identified and quantified with a Fuji phosphorimager and were usually also quantified by excision from the plates and scintillation counting as above.

Subcellular distribution of phosphatidylbutanol in BHK cells

Cells grown to confluence with [3 H]acetate (10 μ Ci/ml) for 48 h in 75 cm² flasks were washed with GMEM and incubated for 30 min at 37 °C in 10 ml of the same medium with 0.2% (v/v) n-butanol with or without the addition of 100 nM PMA. The medium was removed and the cells were washed three times with 10 ml of a 10 mM MOPS/NaOH buffer, pH 7.4, containing 137 mM NaCl, 5 mM KCl and 1 mM MgCl₂. The cells were drained then scraped from the flasks, at 0 °C, homogenized in 1 ml of the same buffer with a 1 ml syringe with a 25 gauge needle (30 strokes) and centrifuged at 500 g_{av} for 5 min at 4 °C to sediment nuclei and incompletely broken cells. The low-speed supernatant (1 ml) was fractionated on a 15–55% (w/w) sucrose density gradient (volume 10 ml and containing the above buffered salt medium) in the 6 $\times$ 11 ml swingout rotor of a Kontron Centrifon D-2190 centrifuge run for 2 h at 150 000 g_{av} . Fractions (0.5 ml) were collected from the top of the gradient with an automatic pipette.

Figure 1 Separation of phosphatidylalcohols by TLC

Lipids were extracted from BHK cells incubated for 20 min at 37 °C alone (lane A), in the presence of 1% (v/v) ethanol/100 nM PMA (lane B) or in the presence of 0.2% (v/v) n-butanol/100 nM PMA (lane C). Samples were separated and detected in a phosphorimager as described in the Materials and methods section. Abbreviations: Ptd, phosphatidyl; PtdOH, phosphatidic acid; Eth, ethanolamine; Ins, inositol; Ser, serine.

Enzyme assays

Alkaline phosphodiesterase, NADPH:cytochrome *c* reductase, β -hexosaminidase and galactosyltransferase [enzyme markers for plasma membrane, endoplasmic reticulum (ER), lysosomes and Golgi respectively] were assayed by standard procedures [18–21].

RESULTS

When BHK cells were labelled to equilibrium with [3 H]choline and then washed and incubated in medium containing non-radioactive choline, there was a steady release of radioactivity into the medium occurring largely at the expense of PtdCho, which declined in radioactivity at the rate of approx. 2%/h (Figure 2, top panel). On addition of 100 nM PMA there was a 4–5-fold increase in the rate of release of choline from PtdCho for about 30 min and then release reverted to the original control rate (Figure 2, top panel). At least 95% of the extracellular radioactivity was shown to be free choline, and more than 75% of the intracellular aqueous pool was found to be phosphocholine after paper chromatography of samples from these aqueous fractions (Figure 2, middle panel). It was noticeable that in the presence of PMA, free choline within the cells rose within 5 min to a steady value of approx. 4% of total radioactivity but that extracellular choline rose more steadily to a final value of about 8% of the total. No significant changes were observed in the radioactivity of sphingomyelin or phosphocholine. The effect of PMA was maximal at approx. 100 nM (Figure 3) and under optimal conditions the total PMA-sensitive release of choline into the cells and medium was equivalent to approx. 10% of total cellular choline or approx. 14% of PtdCho in 30 min.

These findings implied that PMA stimulated a PLD that

Figure 2 Time course of choline release (top and middle panels) and phosphatidylbutanol production (bottom panel) in BHK cells incubated with or without PMA

Top panel: cells were labelled with [^3H]choline and incubated for various times with (●, ■) or without (□, ▽, △, ○) 100 nM PMA as described in the Materials and methods section. Radioactivity in PtdCho (□, ■), sphingomyelin (△), the intracellular aqueous fraction (▽) and the extracellular medium (●, ○) was measured for each time point and the results are expressed as percentages of total radioactivity in each fraction. Similar results were obtained in two other experiments. Middle panel: samples of the medium (○, △) and cell aqueous fractions (▲, ●) from the experiment shown in the upper panel were separated by paper chromatography and the radioactivity of choline (○, ●) and phosphocholine (▲, △) was measured as described in the Materials and methods section. Results are expressed as percentages of total radioactivity in each fraction. Similar results were obtained in two other similar experiments. Bottom panel: cells were labelled to equilibrium with [^3H]acetate and incubated as in the experiment shown in the upper panel with (●) or without (○) 100 nM PMA but with the addition of 0.2% (v/v) n-butanol. Values represent the percentages of radioactivity in phosphatidylbutanol relative to PtdCho and are the means of duplicates that differed by no more than 10%. Similar results were obtained in two other similar experiments.

attacked PtdCho and this was confirmed by using cells labelled to equilibrium with [^3H]acetate, where there was an accumulation of phosphatidylethanol and a decrease in the amount of PtdCho

Figure 3 Dependence of choline release from BHK cells on PMA concentration

Cells were labelled with [^3H]choline, and choline efflux into the medium was measured after incubation for 20 min with various concentrations of PMA as described in the Materials and methods section. Similar results were obtained in a second identical experiment.

in those samples treated with ethanol and PMA (Table 1). Even in the absence of PMA, ethanol caused an increase in phosphatidylethanol that was approx. 10% of that in the presence of PMA and ethanol. With PMA in the absence of ethanol, no significant changes in PtdCho, phosphatidate or phosphatidylethanol were seen (Table 1). With PMA and ethanol present, the accumulation of phosphatidylethanol at 30 min reached only a maximum of approx. 3% of the total PtdCho, whereas loss of label from PtdCho was approx. 8% (Table 1). No significant change was seen in the relative amount of any other lipid, so it seemed that the PLD activity was specific for PtdCho (Table 1). Similar results were seen when butanol was substituted for ethanol (Figures 1 and 2 (bottom panel), and Table 2) except that phosphatidylbutanol production accounted for a larger proportion of the loss from PtdCho (approx. 6% of the total PtdCho). n-Butanol was used in subsequent experiments because it generally gave a larger yield of the phosphatidylalcohol, and the separation from other lipids was better. A time course of phosphatidylbutanol production (Figure 2, bottom panel) was similar to that of choline release (Figure 2, middle panel).

To determine whether PtdCho breakdown was occurring in the plasma membrane, the relative amount of phosphatidylbutanol was measured in whole cells treated with butanol and PMA and in plasma membrane vesicles shed from these cells when they were treated with MC540 and iodoacetamide. As shown previously [10], the vesicles were enriched in the characteristic plasma membrane lipid markers cholesterol, sphingomyelin and phosphatidylserine (Table 2). The relative content of phosphatidylbutanol was considerably greater in the cells (2.5% of total lipid) than it was in the plasma membrane vesicles (1%), suggesting that the primary site of phosphatidylbutanol production could not have been the plasma membrane but was likely to have been some intracellular membrane. Similar results were obtained with HL60 cells exposed to ethanol and PMA and then vesiculated as above (results not shown).

These findings also indicated that, at least during the period of the experiment, phosphatidylbutanol did not equilibrate between cellular membranes; this raised the possibility that subcellular fractionation might be able to reveal the site at which

Table 1 Changes in distribution of radioactivity between the lipids of [3 H]acetate-labelled BHK cells treated with ethanol and/or PMA

[3 H]Acetate-labelled cells were incubated for up to 30 min at 37 °C either with no further addition to the medium or with the addition of PMA (100 nM) and/or ethanol (1%, v/v) as described in the Materials and methods section. Lipids were extracted and separated and then analysed on a Fuji phosphorimager. Results are means $\pm$ S.D. for triplicate determinations and are expressed as percentages of total lipid radioactivity. The decrease in radioactivity in PtdCho in the presence of ethanol and PMA compared with the 30 min control is approx. 9% ($P < 0.002$).

	Lipid proportion (% of total)				
	Control, 0 min	Control, 30 min	With PMA, 30 min	With ethanol, 30 min	With PMA and ethanol, 30 min
Phosphatidylethanol	0.09 $\pm$ 0.01	0.08 $\pm$ 0.01	0.09 $\pm$ 0.02	0.17 $\pm$ 0.02	1.05 $\pm$ 0.1
Triacylglycerol	3.2 $\pm$ 0.2	3.4 $\pm$ 0.6	3.3 $\pm$ 0.5	3.4 $\pm$ 0.1	3.5 $\pm$ 0.1
Cholesterol	18.6 $\pm$ 0.3	19.4 $\pm$ 0.3	19.5 $\pm$ 0.4	19.6 $\pm$ 0.2	19.4 $\pm$ 0.3
Phosphatidic acid	1.5 $\pm$ 0.1	1.6 $\pm$ 0.1	1.9 $\pm$ 0.2	2.0 $\pm$ 0.1	1.8 $\pm$ 0.3
Cardiolipin	4.1 $\pm$ 0.5	4.5 $\pm$ 0.1	4.2 $\pm$ 0.3	4.6 $\pm$ 0.1	4.5 $\pm$ 0.3
Phosphatidylethanolamine	18.1 $\pm$ 0.3	18.6 $\pm$ 0.3	18.5 $\pm$ 0.5	18.7 $\pm$ 0.2	19.0 $\pm$ 0.5
Phosphatidylinositol	5.8 $\pm$ 0.9	5.2 $\pm$ 0.3	5.6 $\pm$ 0.5	5.3 $\pm$ 0.3	5.6 $\pm$ 0.3
Phosphatidylserine	6.6 $\pm$ 0.2	6.6 $\pm$ 0.3	6.7 $\pm$ 0.5	6.8 $\pm$ 0.3	6.8 $\pm$ 0.4
Phosphatidylcholine	35.2 $\pm$ 0.4	34.8 $\pm$ 0.9	34.9 $\pm$ 0.7	33.6 $\pm$ 0.6	32.0 $\pm$ 0.6
Sphingomyelin	5.8 $\pm$ 0.5	6.1 $\pm$ 0.3	6.3 $\pm$ 0.4	6.2 $\pm$ 0.2	5.6 $\pm$ 0.2

Table 2 Distribution of lipids between BHK cells treated with butanol and PMA and plasma membrane vesicles derived from the cells

[3 H]Acetate-labelled cells were incubated for 30 min at 37 °C in the presence of 0.2% (v/v) *n*-butanol with or without addition of 100 nM PMA. The cells were subsequently incubated with merocyanine 540 and iodoacetamide to cause the release of plasma membrane vesicles. Lipids were extracted from vesicles and residual cells, separated and analysed on a Fuji phosphorimager as described in the Materials and methods section. Values are means $\pm$ S.D. for triplicate determinations and are expressed as percentages of total lipid radioactivity. The total mean radioactivities (d.p.m. per dish) were: cells with butanol, 650277 $\pm$ 28499; cells with butanol and PMA, 711342 $\pm$ 25767; vesicles from cells with butanol, 23860 $\pm$ 1645; vesicles from cells with butanol and PMA, 20073 $\pm$ 987.

	Lipid proportion (% of total)			
	Cells treated with		Vesicles from cells treated with	
	Butanol	Butanol and PMA	Butanol	Butanol and PMA
Phosphatidylbutanol	0.43 $\pm$ 0.11	2.46 $\pm$ 0.21	0.47 $\pm$ 0.13	1.03 $\pm$ 0.23
Cholesterol	20.1 $\pm$ 1.9	19.9 $\pm$ 2.3	40.9 $\pm$ 3.4	39.5 $\pm$ 2.7
Phosphatidylethanolamine	18.4 $\pm$ 2.6	17.9 $\pm$ 2.1	8.2 $\pm$ 1.1	7.5 $\pm$ 1.7
Phosphatidylinositol	4.2 $\pm$ 0.9	4.4 $\pm$ 1.3	1.1 $\pm$ 0.8	1.3 $\pm$ 1.1
Phosphatidylserine	4.4 $\pm$ 1.5	4.6 $\pm$ 0.8	10.2 $\pm$ 1.6	11.7 $\pm$ 1.4
Phosphatidylcholine	42.3 $\pm$ 2.4	38.6 $\pm$ 1.8	23.2 $\pm$ 2.9	22.9 $\pm$ 2.2
Sphingomyelin	5.3 $\pm$ 0.9	5.6 $\pm$ 0.8	15.5 $\pm$ 2.1	16.5 $\pm$ 1.5

phosphatidylbutanol was produced initially. Separation of the low-speed supernatant from a homogenate of [3 H]acetate-labelled cells on a sucrose density gradient (Figure 4a) revealed three peaks of phosphatidylbutanol concentration, a low-density peak (I) near the top of the gradient and peaks II and III at relative densities of 1.13 and 1.17 (Figure 4b) respectively. Peak I contained hexosaminidase activity and corresponded to material associated with the 'floating lysosomes' previously reported in BHK cells [22–24]. Peak II migrated close to the peaks of cholesterol, sphingomyelin (Figure 4c) and phosphatidylserine (results not shown), which are generally considered to be the characteristic lipids of plasma membranes. Alkaline phosphodiesterase, an enzymic marker of plasma membranes, also showed a

peak in this region (Figure 4d). The peak of phosphatidylbutanol concentration of highest density, peak III, coincided with a shoulder on the profile of PtdCho radioactivity but was relatively poor in phosphatidylserine, sphingomyelin and cholesterol. Phosphatidylinositol was the phospholipid that showed the best correspondence to phosphatidylbutanol in terms of its distribution across the gradient (Figure 4b). Significantly, a similar triphasic distribution was seen for the activity of cyanide-insensitive NADPH:cytochrome *c* reductase, generally regarded as a marker for the ER (Figure 4b). In contrast there was no apparent relationship between the distribution of phosphatidylbutanol and the Golgi marker, galactosyltransferase, which migrated in the region of relative density 1.08 (Figure 4d).

DISCUSSION

Degradation of PtdCho by PLD in intact cells

On the basis of the observed generation of choline (Figure 2) and the production of phosphatidylethanol (Table 1) in the absence of PMA it seems that BHK cells possess a PLD activity that in normal cells causes the breakdown of approx. 2%/h of total PtdCho. It is not clear whether this endogenous PLD accounts for all the loss of label from PtdCho or whether other mechanisms (e.g. base exchange) could also contribute.

In the presence of PMA, the breakdown of PtdCho and the accumulation of phosphatidylbutanol were increased approx. 5-fold, with choline being released initially inside the cells (Figure 2, middle panel) but rapidly appearing in the medium. This behaviour is expected because the transporter protein that normally imports choline into the cells should act to mediate the efflux of radioactive choline from the cells. The enhanced efflux of radioactive choline ceased after approx. 30 min, when efflux reverted to the original value seen in untreated cells (Figure 2, top panel). At this point a maximum of approx. 12% of the total radioactivity in PtdCho had been lost as choline. This seems likely to represent the relative mass of PtdCho that was degraded, because the cells were labelled to equilibrium with [3 H]choline and then chased in medium containing non-radioactive choline, which equilibrates rapidly with intracellular pools. The chase is likely to be less effective with [3 H]acetate-labelled cells, and the

Figure 4 Subcellular fractionation of [^3H]acetate-labelled BHK cells on a sucrose density gradient

[^3H]Acetate-labelled cells were treated with PMA and fractionated on a 15–55% (w/w) sucrose density gradient as described in the Materials and methods section. The top of the gradient is on the left. (a) The relation between density and fraction number. (b) The distribution of phosphatidylbutanol (●), phosphatidylinositol (○) and cyanide-insensitive NADPH:cyto-

possibilities here for reutilization of label to resynthesize PtdCho could explain the somewhat lower nominal figures for PtdCho degradation (8–10%). With PMA alone, no significant change in the total PtdCho and only a minimal increase in phosphatidate were seen (Table 1), suggesting that in the absence of alcohols there is a rapid resynthesis of PtdCho that has been degraded by PLD.

Values for PtdCho degradation based on phosphatidylbutanol or phosphatidylethanol production were generally lower than those based on loss of label from PtdCho, although with n-butanol approx. 60% of the PtdCho lost could be accounted for as phosphatidylbutanol (Table 2). In contrast with a previous report on PMA-stimulated PLD in BHK cells [13], no significant effect was seen on phosphatidylethanolamine or any other lipid apart from PtdCho (Tables 1 and 2), although it would have been difficult to detect if a small proportion of the lipid backbone derived from PtdCho was degraded or utilized for general lipid biosynthesis.

The simplest explanation for the apparent limitation on the PMA-dependent breakdown of PtdCho (Figure 2, top panel) is that the PLD has access to only a small pool of PtdCho that cannot rapidly be refilled at the expense of larger PtdCho pools in the cell. Alternatively, it might be that the PLD is rapidly inactivated, perhaps by a desensitization of the target protein kinase C, which is presumed to activate the PLD. It could be coincidence that the rate of release of choline after 30 min of treatment with PMA is similar to that in unstimulated cells (Figure 2, top panel) or it could be an indication that there is an endogenous PLD activity (or base-exchange process) that is not affected by PMA and operates at its original rate after PMA has had its brief effect on the sensitive pool of PtdCho.

Localization of PtdCho degradation

Most of the accumulation of phosphatidylbutanol in PMA-treated cells seems not to be in the plasma membrane, from a comparison of the amount of phosphatidylbutanol in intact cells with that in plasma membrane vesicles. Thus the relative change in phosphatidylbutanol concentration in intact cells was approx. 5-fold whereas it was only approx. 2-fold in plasma membrane vesicles. Given that plasma membrane accounts for approx. 30% of the lipids of the intact cells [12] it can be calculated from these values that phosphatidylbutanol must account for approx. 3% of the lipids of the internal membranes, compared with only 1% of the lipids of the plasma membrane vesicles. This seems to exclude the plasma membrane as the primary site of the PMA-sensitive PLD activity because the quantity of phosphatidylbutanol in the vesicles is so much smaller than in the intact cells or internal membranes. There could, however, be a minor site of PMA-sensitive PLD activity in plasma membrane.

The results of cell fractionation on a density gradient might also be consistent with the presence of some PLD in the plasma membrane because one of the peaks of phosphatidylbutanol is close to that of the plasma membrane markers (relative density approx. 1.13). However, the fact that ER markers including NADPH:cytochrome *c* reductase and phosphatidylinositol [12]

chrome *c* reductase (■) on the density gradient. The real values for phosphatidylbutanol are multiplied by 2 to enhance the comparison with phosphatidylinositol. The units for NADPH:cytochrome *c* reductase are arbitrary, on the basis of A_{550} . (c) Density gradient distribution of PtdCho (○), sphingomyelin (■) and cholesterol (▼). (d) Density gradient distribution of β -hexosaminidase (■), galactosyltransferase (○) and alkaline phosphodiesterase (▲). Similar results were obtained in two further experiments.

show very similar distributions to that of phosphatidylbutanol suggests the alternative idea that if the denser peak corresponds to rough ER, then the lighter peak close to the plasma membrane region could be smooth ER. Relatively little of the phosphatidylbutanol need then be assigned to the plasma membrane and indeed the levels found in the plasma membrane vesicles could be an overestimate owing to the fact that the vesicles are not pure [10,14] and to the possibility that the period of incubation at 37 °C with merocyanine 540 and iodoacetamide might allow some movement of phosphatidylbutanol from the ER to the plasma membrane, which does not occur during cell fractionation at low temperature.

Our conclusion is therefore that because phosphatidylbutanol distribution on the density gradient largely follows ER markers (Figure 4b), then PMA-sensitive PLD in BHK cells is acting mainly on PtdCho in the ER. Using a similar labelling technique in HeLa cells, Edwards and Murray [8] also showed the production of phosphatidylalcohol in ER-enriched fractions, although in this case a similar amount was also found in the plasma membrane fraction. These authors noted that 'it is widely assumed that PLD activated by PKC during signal transduction is associated with the plasma membrane' but evidently PMA-sensitive (and thus presumably PKC-activated) PLD can also reside in the ER. Recently, PLD activity has been demonstrated in nuclei [25], presumably in the nuclear membrane, which is closely related to the ER and resembles it in lipid composition [26]. There is also a precedent for an association of PLD with the Golgi apparatus [27] but the present work lends no support to the idea that the PMA-sensitive PLD is enriched in the Golgi in BHK cells; no peak of PLD activity corresponded to Golgi markers on the sucrose density gradient (Figures 4a and 4d). There was an apparent association of phosphatidylbutanol with a light peak on the gradient that contains lysosomal markers (Figures 4b and 4d) but it is not clear from the present results whether it represents a specific association of PLD activity with lysosomes or whether this fraction is a repository for degraded membranes exhibiting PLD activity from other sites in the cell.

It is worth noting that labelling lipids to equilibrium with [³H]acetate provides what are effectively lipid mass markers that can be used to characterize cell subfractions. Thus it seems from the present work that cholesterol, sphingomyelin and phosphatidylserine (results not shown) are useful markers for the plasma membrane, and that phosphatidylinositol is a marker for ER (Figures 4b and 4c).

ER seems to account for approx. 40 % of the PtdCho of BHK cells [12], whereas the maximum measured loss of [³H]choline radioactivity from PtdCho in cells treated with PMA was only approx. 14 % (Figure 2). It is not obvious why breakdown seems to be limited to only about one-third of the total PtdCho in the ER unless, as noted above, there is a rapid inactivation of PLD after 30 min. Even if the PLD can access only the cytoplasmic leaflet of the ER lipid bilayer, which accounts for somewhere

between 55 % [28] and 72 % [29] of microsomal PtdCho, breakdown still represents only approx. one-half of the PtdCho in this leaflet. Although the luminal pool of PtdCho in the ER could be similar in size to the pool that is degraded by PLD, it seems unlikely that this is the site of action of the PMA-sensitive PLD. Quite apart from the conceptual difficulties in imagining how a luminal PLD could be activated physiologically, the rapid exit of choline from the cells (Figure 2, middle panel) suggests that it must be generated by breakdown of PtdCho on the cytosolic face of a membrane.

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