Accumulation of phosphatidylalcohol in cultured cells: use of subcellular fractionation to investigate phospholipase D activity during signal transduction

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Phosphatidylalcohol accumulates as a product of a phospholipase D (PLD)-catalysed transphosphatidylation reaction in cells incubated in the presence of a primary alcohol. In the presence of ethanol the phorbol ester, phorbol 12-myristate 13-acetate (PMA), stimulated the accumulation of [^{3}H]phosphatidylethanol (PEth) in HeLa cells prelabelled with [^{3}H]palmitic acid. Radioactivity associated with PEth increased linearly during a 30 min incubation, indicating that a sustained activation of PLD is caused by PMA in these cells. This was accompanied by the membrane association of protein kinase C-α (PKC-α), the PKC isoform that recent studies indicate is involved in the activation of PLD. In similar experiments, the neuropeptide bradykinin stimulated an accumulation of PEth in 3T3 Li cells. The radioactivity associated with PEth increased to a maximal level at 30 s and plateaued after this time, suggesting that bradykinin induces only a transient activation of PLD in these cells. This is consistent with the effects of bradykinin on PKC-α, which underwent a rapid and transient association with cell membranes. The subcellular localization of PEth was examined using the technique of subcellular fractionation on Percoll density gradients to isolate organelle-enriched fractions from HeLa and 3T3 Li cells. An accumulation of [^{3}H]PEth was measured in the plasma-membrane (PM)-enriched fractions of both HeLa and 3T3 Li cells after incubation with PMA and bradykinin respectively. This was accompanied by a time-dependent accumulation of [^{3}H]PEth in the combined mitochondrial and endoplasmic reticulum (MER)-enriched fractions of both cell lines. PMA was also found to cause translocation of PKC-α to both the PM- and MER-enriched fractions in HeLa cells. However, bradykinin stimulated the translocation of PKC-α to the PM-enriched fractions only of 3T3 Li cells. The results show that PLD activation leads to the accumulation of PEth in both the PM and MER fractions. We therefore propose that either bradykinin activates a PM-associated PLD and the PLD reaction product is rapidly translocated to other membrane systems or it activates an MER-associated PLD by a mechanism that does not involve PKC-α.

INTRODUCTION

It is now well established that many agonists stimulate signalling pathways which involve the generation of lipid-derived second-messenger molecules. One of the most extensively studied pathways is that involving the hydrolysis of PtdInsP_2 by phospholipase C (PLC). This is activated by many Ca^{2+}-mobilizing stimuli and leads to the transient accumulation of the two second-messenger molecules, InsP_3, which releases Ca^{2+} from intracellular stores [1], and diacylglycerol (DG), which activates protein kinase C (PKC) [2]. Recent studies indicate that the hydrolysis of PtdCho via phospholipase D (PLD) is also a major signalling pathway stimulated by many agonists [3]. The activation of PLD can occur through either direct receptor stimulation or a PKC-dependent mechanism. This leads to the formation of PtdOH which is readily converted into DG by subsequent activation of PtdOH phosphohydrolase [4]. Evidence suggests that both of these PtdCho-derived molecules are involved in regulating the downstream activity of various enzymes including PKC (for a review see [5]), and it is therefore likely that PtdCho functions as an important source of second-messenger molecules during signal transduction.

The stimulation of hydrolysis of PtdInsP_2 and PtdCho is generally measured by analysing the increase in radioactivity associated with PtdOH and DG products after radioactive prelabelling of membrane phospholipid [6]. This is often carried out in parallel with measurements of lipid mass and fatty acid composition which are used to clarify the relative contributions of PtdInsP_2 and PtdCho turnover to second-messenger accumulation [7–9]. However, a fundamental limitation of most of these studies is that measurements are carried out on lipids extracted from whole cells. Under these conditions it is difficult to identify the PtdOH and DG pools that are specifically derived from plasma-membrane phospholipids after cell stimulation. Indeed many agonists stimulate the rapid *de novo* synthesis of lipids [6]. This would cause significant increases in PtdOH and DG levels in the endoplasmic reticulum which would therefore contribute to the total PtdOH and DG measured from whole cells. Furthermore, it is impossible to determine from whole-cell studies whether second-messenger molecules such as PtdOH and DG remain associated with the plasma membrane immediately after their generation at this site. Studies by Pagano and Longmuir [10], for example, suggest that DG derived from plasma-membrane-associated fluorescently labelled PtdCho undergoes rapid translocation to intracellular membranes in fibroblasts. There is also increasing evidence that DG-dependent enzymes such as PKC and CTP–phosphocholine cytidylyltransferase are activated after receptor-linked translocation to intracellular sites [11,12]. It is therefore possible that the subcellular relocation of DG and perhaps PtdOH after their initial generation on the plasma

Abbreviations used: DG, diacylglycerol: lyso-PAF, lyso-platelet-activating factor; MER I, mitochondrial/endoplasmic reticulum I; MER II, mitochondrial/endoplasmic reticulum II; PEth, phosphatidylethanol; PBut, phosphatidylbutanol; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PM, plasma membrane; PMA, phorbol 12-myristate 13-acetate.

membrane could be an integral part of their second-messenger function during signal transduction.

In this paper we report a protocol that can be used to determine the subcellular localization of lipid-derived second-messenger molecules. The technique of subcellular fractionation is used to isolate organelle-enriched fractions which are then analysed for various lipids after cell stimulation. In the initial series of experiments reported here the subcellular accumulation of lipid products after PLD-mediated PtdCho hydrolysis was examined. The activation of PLD by phorbol 12-myristate 13-acetate (PMA) and bradykinin in HeLa and 3T3 Li cells respectively was assayed by measuring the accumulation of phosphatidylalcohol, which is a relatively stable product of a PLD-catalysed transphosphatidylation reaction [13]. The subcellular distribution of PKC-α, the PKC isoform that recent studies indicate is involved in the activation of PLD [14], was also analysed in these cells.

MATERIALS AND METHODS

Materials

[9,10-^{3}H]Palmitic acid (54 Ci/mmol), [*methyl*-^{3}H]choline (74.4 Ci/mmol) and 1-*O*-[^{3}H]octadecyl-*sn*-glycerophosphocholine (lyso-PtdOH; 163 Ci/mmol) were obtained from Amersham Corp. (Amersham, Bucks., U.K.). Percoll was from Pharmacia LKB Biotechnology, and precoated silica-gel TLC plates were from Merck. PLD (cabbage) from Sigma was used to prepare phosphatidylethanol (PEth) and phosphatidylbutanol (PBut) standards as described [15]. PMA was from P-L Biochemicals, and bradykinin was from Sigma. Peptide-purified PKC-α as well as the corresponding peptide were from Boehringer, and sheep anti-rabbit IgG–horseradish peroxidase conjugate was from Silenus. Supported nitrocellulose membrane (0.5 μM) was from Schleicher and Schuell, and enhanced chemiluminescence reagents were from NEN–Dupont.

Cell culture and labelling

Both HeLa cells (ATCC CCL2) and 3T3 Li cells (ATCC CCL92.1) were obtained from the American Type Tissue Collection and were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Cells were grown in 35 mm dishes for whole-cell lipid studies and in 150 mm dishes for all other experiments. Subconfluent HeLa and 3T3 Li cells were prelabelled for 24 h with [^{3}H]palmitic acid (1 μCi/ml) in normal and starvation medium (1% fetal calf serum) respectively. The medium was then removed and the cells were washed twice with PBS before being incubated with test substances as indicated. For choline-incorporation studies, HeLa cells were incubated with [^{3}H]choline (10 μCi/dish) for 30 min.

Subcellular fractionation

Fractionation procedures were carried out at 4 °C and were based on the original method of Record and co-workers [16]. Cells were harvested by trypsinization from three 150 mm dishes (HeLa cells approx. 18×10^6; 3T3 Li cells approx. 9×10^6) after incubation with test substances. The cells were washed once with PBS and once with isotonic lysis buffer at pH 9.6 (100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 10 mM benzamidine, 2 mM PMSF and 25 mM Tris/HCl). The washed cell pellet was resuspended in 5 ml of lysis buffer and disrupted with a tight-fitting Dounce homogenizer (100 strokes). Unbroken cells were pelleted by centrifugation (200 *g*; 10 min), resuspended in lysis buffer and subjected to a second round of disruption followed by centrifugation. The 200 *g* supernatants were combined and centrifuged at 1000 *g* for 5 min to pellet nuclei and cell debris. The supernatant was used as the total cell lysate and was centrifuged at 100000 *g* for 60 min to pellet a crude membrane fraction. The pellet was resuspended in gradient solution containing 5.5 ml of Percoll, 1.1 ml of distilled water, 2 ml of lysis buffer and 2.4 ml of buffer comprising 400 mM KCl, 20 mM $MgCl_2$ and 100 mM Tris/HCl, pH 9.6. The final pH was carefully monitored and readjusted to 9.6 with concentrated Tris when necessary. The gradient solution was centrifuged in a Sorval F28/13 rotor at 38000 *g* for 33 min (corresponding to an $\omega^2 t$ of 1.29×10^{10} rad^2/s). Fractions (0.5 ml) were collected from the bottom of the tube, adjusted to pH 7.4 with 0.25 ml of buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM benzamidine, 2 mM PMSF and 50 mM Tris/HCl, pH 7.4) and stored at 4 °C. Marker enzyme assays, protein determinations, lipid analysis and Western-blot analysis were carried out as described below.

Subcellular membrane preparation

Plasma membrane (PM)- and mitochondrial/endoplasmic reticulum I/II (MER I/II)-enriched fractions were pooled, diluted to 10 ml with membrane buffer (2 mM EDTA, 5 mM EGTA, 0.001% leupeptin, 10 mM benzamidine, 2 mM PMSF, 10 mM mercaptoethanol and 20 mM Tris/HCl, pH 7.4) and centrifuged at 100000 *g* for 45 min. Membranes were collected at the Percoll/buffer interface and resuspended by sonication (3×5 s) in 900 μl of membrane buffer. Aliquots were either used directly for subcellular lipid analysis or incubated with Triton X-100 for the preparation of membrane extracts as outlined below.

Lipid analysis

For whole-cell studies, incubations with test substances were terminated by removal of the medium and the addition of 2 ml of cold methanol. Lipids were extracted as described [17]. In subcellular-fractionation studies, lipids were extracted from either 25 μl aliquots of each gradient fraction or 100 μl aliquots of either PM or MER I/II membrane suspensions. PEth was separated by TLC as described [18], and PtdCho was separated by two-dimensional TLC [17]. Lipids were visualized by iodine staining, and the radioactivity associated with PEth and PBut was determined by liquid-scintillation spectrometry after scraping samples from TLC plates. Labelled PtdCho in the subcellular fractions of cells prelabelled with [^{3}H]palmitic acid was distributed equally between the PM and MER (combined MER I/II) membranes (results not shown). In subcellular-fractionation studies results were expressed as the total amount of radioactivity associated with PEth per fraction. During the course of incubations with PMA and bradykinin, the radioactivity associated with total phospholipid increased in the MER fraction and decreased in the PM fraction. This is presumably the result of stimulation of *de novo* synthesis in the ER [12] and activation of phospholipid hydrolysis in the PM. However, similar peak positions and stimulation trends were observed when the results were expressed as radioactivity accumulated in PEth as a percentage of phospholipid radioactivity in unstimulated fractions.

Preparation of membrane extracts

Detergent extracts were prepared from total cell membranes by resuspending the crude membrane pellet obtained after cell disruption in membrane buffer containing Triton X-100 (2%). Detergent extracts from PM and MER I/II membranes were

obtained by adding Triton X-100 (2%) to membrane suspensions prepared after subcellular fractionation. Incubations were carried out for 60 min at 4 °C and the Triton-soluble proteins (supernatant) collected after centrifugation at 100000 *g* for 20 min. Aliquots mixed with an equal volume of Laemmli sample buffer [19] were heated for 5 min at 100 °C and used for Western-blot analysis.

Western-blot analysis

Proteins (20–30 μg) were separated by electrophoresis as described by Laemmli [19] on SDS/polyacrylamide gels (12% polyacrylamide; 200 V, 0.8 h; Mini-Protein II gel system; Bio-Rad). After electrophoresis, gels were pre-equilibrated for 15 min in transfer buffer (25 mM Tris, 152 mM glycine, 1.3 mM SDS and 20% methanol) after which proteins were transferred to nitrocellulose membranes (100 V, 1.5 h; Mini Transfer system; Bio-Rad). After transfer, membranes were incubated with blocking solution (0.1% Tween 20, 5% skimmed-milk powder, 40 mM Tris/HCl, pH 7.4) for 60 min at room temperature followed by a further 60 min at 37 °C with renewed blocking solution containing peptide-purified PKC-α antibody (40 μg/ml). Membranes were rinsed in washing solution (0.1% Tween 20, 5% skimmed-milk powder, 0.15 M NaCl, 20 mM Tris/HCl, pH 7.4) and incubated in blocking solution containing goat anti-rabbit IgG–horseradish peroxidase conjugate (1:1000 dilution) for 30 min at 37 °C. Membranes were again rinsed in washing solution and immunoreactive bands were detected using enhanced chemiluminescence according to the manufacturer's protocol.

Subcellular markers

Rotenone-insensitive NADH–cytochrome *c* reductase activity was determined by the method of Sottocasa and co-workers [20] and was used to identify the MER fraction. The PM marker used was 5′-nucleotidase and was assayed by a method adapted from that of Evans [21]. Briefly, activity was assayed in 0.5 ml of incubation medium containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM AMP and 50 mM Tris/HCl, pH 8.5. The reaction was started by adding 100 μl of the gradient fraction and stopped after 30 min with 10 μl of perchloric acid. Liberated phosphate was measured as described [22]. The incorporation of [^{3}H]choline into PtdCho by *de novo* synthesis after a 30 min pulse with the label was used as an additional marker for the ER.

Protein determination

Protein was determined by the method of Bradford [23].

Statistical analysis

Results were analysed by Student's unpaired *t* test.

RESULTS AND DISCUSSION

A subcellular fractionation procedure using Percoll density gradients was used to isolate organelle-enriched fractions from HeLa and 3T3 Li cells. A key feature of this protocol is that cell disruption, centrifugation and fractionation are carried out at a pH of 9.6. The selection of this pH is based on evidence that alkaline conditions can cause a selective increase in the density of ER vesicles resulting in an improved separation from PM fragments on a density gradient [24]. Recent work has shown that this improved separation may also be partly due to the dissociation of ER from PM caused by the disruption of cytoskeletal links during alkaline treatment [25,26]. As shown in Figure 1(a), ER sediments close to the mitochondria at pH 9.6, generating two fractions variably enriched in these organelles. These are labelled MER I and MER II and are located in the first few fractions of the Percoll gradient as determined by rotenone-insensitive NADH–cytochrome *c* reductase activity. This enzyme is found in both the mitochondria and ER [20] and was therefore routinely used to identify both MER I and MER II. The PM-enriched fractions were identified by 5′-nucleotidase activity; they are clearly separated from MER I and MER II on the density gradient (Figure 1a). The predominant accumulation of [^{3}H]PtdCho in MER I after short-term incubation of cells with [^{3}H]choline indicates that this fraction is enriched in ER (Figure 1b). This is supported by the gradient activity of other marker enzymes including glucose 6-phosphatase (results not shown).

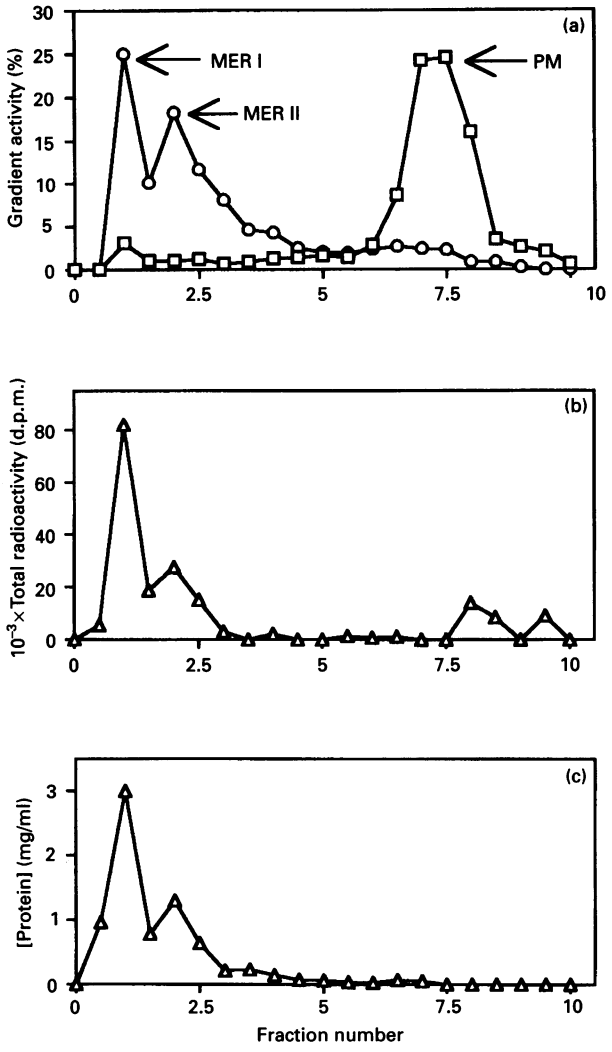

Figure 1 Typical gradient distribution of MER I-, MER II- and PM-enriched fractions from HeLa and 3T3 Li cells

(**a**) Marker enzyme activities, rotenone-insensitive NADH–cytochrome *c* reductase (MER; ○) and 5′-nucleotidase (PM; □), were assayed as described in the Materials and methods section. (**b**) Subcellular accumulation of [^{3}H]PtdCho was measured after a 30 min incubation of cells with [^{3}H]choline, and (**c**) subcellular protein distribution was determined as described in the Materials and methods section.

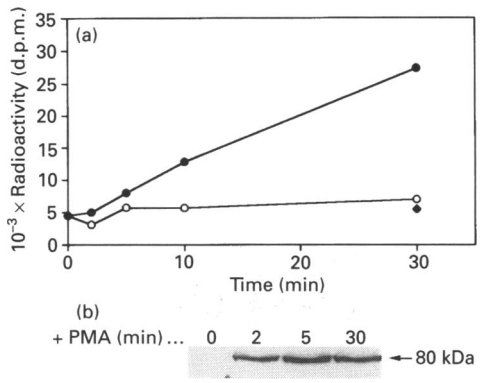

Figure 2 (a) Time course of PEth accumulation and (b) PKC-α translocation in PMA-stimulated HeLa cells

(**a**) Cells plated in 35 mm dishes and prelabelled with [^{3}H]palmitic acid were used for analysis of PEth as described in the Materials and methods section. Lipids were extracted and the radioactivity associated with PEth was determined after incubation of these cells for the indicated times with 0.5% ethanol in the presence of either 0.2% DMSO (○) or 100 nM PMA (●) or with 100 nM PMA alone (◆). Each point represents the mean ± S.E.M. of three replicate determinations. In this Figure the S.E.M. falls within the symbol. (**b**) Cells plated in 150 mm dishes were used to analyse membrane-associated PKC-α. Membrane extracts prepared from cells after the incubation with test substances as outlined above were subjected to Western-blot analysis as described in the Materials and methods section.

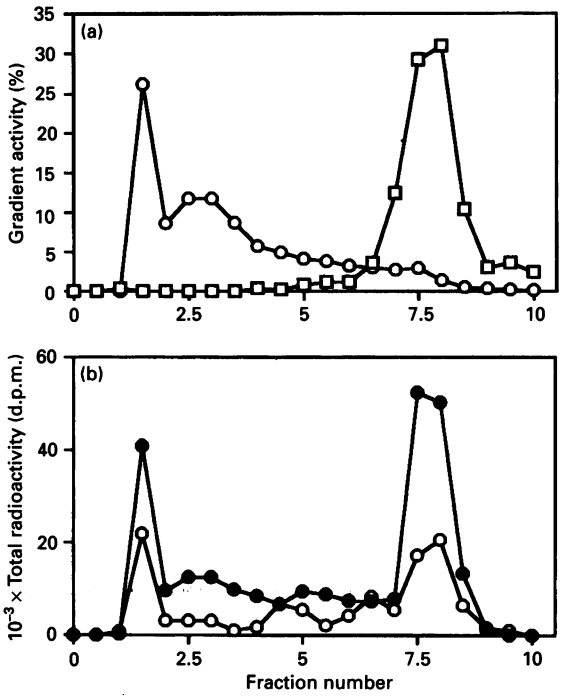

Figure 3 Distribution of PEth in subcellular fractions isolated from PMA-stimulated HeLa cells

Cells prelabelled with [^{3}H]palmitic acid as described in the Materials and methods section were incubated with 0.5% ethanol for 25 min followed by a further 5 min in the presence of either 0.2% DMSO or 100 nM PMA. They were then harvested, disrupted and fractionated on Percoll density gradients, and the fractions analysed for rotenone-insensitive NADH–cytochrome *c* reductase activity (○) and 5′-nucleotidase activity (□) (**a**). Radioactivity associated with PEth was measured for each gradient fraction (○, DMSO; ●, PMA) (**b**). Enzyme assays and lipid analysis were carried out as described in the Materials and methods section. These data are representative of three separate experiments.

Figure 1(c) shows a typical distribution of protein on the Percoll gradient. As reported elsewhere for cultured cells [27], less than 2% of the total protein was associated with the PM, the majority being located in mitochondrial and ER membranes. Marker enzymes for other organelles such as the Golgi and lysosomes were not assayed. The sedimentation characteristics of these organelles under alkaline conditions are also unknown. We have therefore not eliminated the possibility that Golgi and lysosomal vesicles/fragments may also be present in the PM- and MER I/II-enriched fractions.

The formation of PEth, which is generated in the presence of ethanol by the PLD-catalysed transphosphatidylation of PtdCho, is a convenient index of PLD activity. PMA caused a rapid ethanol-dependent accumulation of [^{3}H]PEth in HeLa cells prelabelled with [^{3}H]palmitic acid (Figure 2a). Radioactivity associated with PEth was detectable within 2 min and increased linearly up until 30 min. This accumulation, which continues for at least 120 min (results not shown), indicates that PMA causes sustained activation of PLD in these cells. PMA functions as a structural analogue of DG and stimulates PLD via activation of PKC. PKC is known to exist as a family of at least 12 structurally related isoforms which vary in their subcellular distribution, substrate specificity and regulation by Ca^{2+} and DG [28]. Evidence from *in vitro* [14] as well as *in vivo* studies using transfection with antisense PKC-α [29] suggests that the α-isoform of PKC is involved in the activation of PLD in several cell types. The effect of PMA on PKC-α was therefore investigated in HeLa cells. PKC-α is the major isoform expressed in these cells, with smaller amounts of the ε- and ζ-isoforms as detected in cell extracts by Western-blot analysis using peptide-purified PKC antibodies (results not shown). The identity of the immunoreactive bands was confirmed by competition studies in which the immunoreactivity was blocked by preincubation of the antibody with the specific peptide against which it had been raised (results not shown). Stimulation of cells with PMA caused a rapid and sustained translocation of PKC-α to the membrane fraction (Figure 2b), which paralleled the accumulation of PEth in these cells. Translocation of pKC-ε and PKC-ζ was not examined.

It is widely assumed that the PLD activated by PKC during signal transduction is associated with the PM. The accumulation of PEth after cell stimulation is therefore also expected to occur at this site. To investigate this, the membrane localization of PEth was examined in PMA-stimulated HeLa cells. Figure 3 shows the distribution of subcellular marker enzyme activity and [^{3}H]PEth in membrane fractions obtained after cell fractionation on a Percoll density gradient. Radioactivity was associated with both the MER I- and PM-enriched fractions of unstimulated cells incubated in the presence of ethanol (Figure 3b). This may be due to the formation of [^{3}H]PEth in these fractions by a PLD active in the absence of a stimulus, although we have shown this to be low in whole-cell studies [30]. Alternatively labelled lipids other than [^{3}H]PEth may also be present in the PEth region of the TLC plate. For example, bisphosphatidic acid, which is formed in the presence of DG by a PLD-catalysed transphosphatidylation reaction, has recently been shown to co-migrate with PEth during lipid separation by TLC [31]. However, in a separate series of experiments, the radioactivity associated with PBut was measured, which is generated instead of PEth after the stimulation of cells in the presence of butanol. In these experiments, radioactivity was detected in the PBut region of the TLC plate during lipid analysis of the subcellular fractions of unstimulated cells (results not shown). The contribution of lipid

Table 1 Time course of PEth accumulation in MER and PM subcellular fractions of PMA-stimulated HeLa cells

Cells prelabelled with [^{3}H]palmitic acid for 24 h were incubated with 100 nM PMA for the indicated times during a 30 min incubation with 0.5% ethanol. The PM- and MER (combined MER I/II)-enriched fractions were isolated on a Percoll density gradient, and the total radioactivity associated with PEth was determined as described in the Materials and methods section. Results are expressed as means ± S.E.M. of three replicate determinations. Similar results were obtained in three separate experiments.

	Subcellular distribution of [^{3}H]PEth (total d.p.m.)	
Time (min)	MER	PM
0	10878 ± 326	8031 ± 775
2	18639 ± 1113	12560 ($n = 2$)
5	22926 ± 438	21354 ± 443
30	72465 ± 1389	38160 ± 943

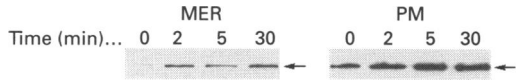

Figure 4 PKC-α translocation to MER and PM subcellular fractions of PMA-stimulated HeLa cells

Cells prelabelled with [^{3}H]palmitic acid for 24 h were incubated with 100 nM PMA for the indicated times during a 30 min incubation with 0.5% ethanol. The PM- and MER (combined MER I/II)-enriched fractions were isolated on a Percoll density gradient. The effect of PMA on PKC-α in these fractions was assessed using Western-blot analysis as described in the Materials and methods section.

molecules such as bisphosphatidic acid to PEth and PBut levels in unstimulated cells is unclear.

Incubation of cells for 5 min with PMA caused an increase in the labelled PEth associated with PM-enriched fractions (Figure 3b) which is consistent with the activation of a PM-associated PLD. PMA also caused an unexpected increase in the labelled PEth associated with MER I and MER II (Figure 3b) which, as shown by the subcellular marker enzyme profiles (Figure 3a), was not due to cross-contamination with the PM. A series of experiments was therefore carried out to investigate the mechanism(s) that may be involved in the accumulation of PEth in these subcellular fractions. In an initial experiment, the effect of PMA on both PEth accumulation and PKC-α translocation was investigated as a function of time. PMA caused a rapid and sustained increase in the [^{3}H]PEth associated with both the MER and PM fractions (Table 1). Accumulation of PEth in the PM is consistent with the activation of a PM-associated PLD and may be mediated by PKC-α which, as shown in Figure 4, was translocated to the PM in response to PMA. However, the mechanism responsible for the dramatic increase in MER-associated PEth is unclear. A series of control experiments was carried out to exclude the possibility that MER-associated PEth was an artifact, generated as a result of transfer from the PM during experimental manipulation. Figure 5 shows the results of mixing experiments which analysed the transfer of both [^{3}H]PEth and PKC-α between the PM and MER fractions of HeLa cells during the processes of cell disruption and fractionation. No transfer of either preformed PEth (Figures 5a–5d) or PKC-α (Figure 5e) occurred between subcellular fractions. This suggests that both the PEth and PKC-α associated with the membrane fractions isolated on the density gradient were there as the result of events that had taken place in the cells before lysis.

PMA and other related phorbol esters have been extensively used in studies of PLD and other PKC-dependent enzymes. As lipophilic molecules they are able to intercalate rapidly into the membranes of cells and stimulate enzyme activity in the absence of any receptor activation. It is therefore possible that the accumulation of PEth in the MER membranes of PMA-stimulated cells (Table 1) is due to the activation of an intracellular PLD isoform not normally accessible to receptor-dependent agonists. This activation, if PKC-dependent, could be mediated by PKC-α which was translocated to MER membranes after PMA stimulation (Figure 4). A series of experiments was carried out to address the possibility that the accumulation of MER-associated PEth after a 30 min exposure to PMA could be due to the activation of a 'late-acting' intracellular PLD isoform. In these experiments slightly more PEth was found to accumulate in the MER membranes than the PM of cells when exposed to ethanol (5–10 min) at the end of a 30 min pretreatment with PMA (results not shown). This is consistent with the existence of an MER-associated PLD which is activated in a delayed fashion in PMA-stimulated HeLa cells. However, stimulatory effects of PMA on intracellular events such as intracellular lipid trafficking cannot be eliminated as a possible explanation for these results.

It is also possible that other factors such as the ADP-ribosylation factor could be involved in the activation of an intracellular PLD isoform. This small GTP-dependent regulatory protein which is involved in the control of intracellular vesicle transport [32,33] has recently been shown to activate PLD [34–36]. A role for PLD in the control of vesicle transport by ADP-ribosylation factor has subsequently been proposed [37,38] and could in fact explain the existence of an intracellular PLD isoform. Work is currently underway to assay the *in vitro* activity of PLD in the PM and MER membranes of cells. It is expected that this approach, which is based on the use of PLD-reconstitution systems [34,39], will help to establish whether an intracellular PLD is involved in the accumulation of MER-associated PEth in PMA-stimulated HeLa cells.

An alternative explanation for the time-dependent accumulation of labelled PEth in MER membranes is that translocation to this site occurs after initial synthesis has taken place in the PM of stimulated cells. To investigate this, a series of experiments was carried out with the aim of using [^{3}H]lyso-platelet-activating factor ([^{3}H]lyso-PAF] to selectively label PEth in the PM. [^{3}H]Lyso-PAF, which is a relatively stable ether-linked form of lyso-PtdCho, is rapidly acylated on the inner leaflet of the PM. It is therefore often used to selectively label the PM-associated pool of PtdCho [30,40] and should enable the translocation of PM-derived PEth to be monitored. However, the results obtained from preliminary experiments (results not shown) indicate that MER membranes have access to an efficient acylation mechanism which rapidly generates labelled PtdCho in the MER fraction of whole cells as well as in broken-cell preparations. The origin of any [^{3}H]PEth that may accumulate in the MER fraction cannot therefore be determined from studies using this labelling protocol.

An alternative approach was therefore chosen to investigate the possibility that PM-associated PEth was able to be translocated to MER membranes. The agonist bradykinin was used which, in contrast with PMA, interacts with cells via cell surface receptors. It is a neuropeptide that is involved in regulating tissue response to trauma and injury, and has been shown to interact with a variety of cells causing the PKC-mediated activation of a Ptd-Cho-specific PLD [41,42]. The activation of PLD was examined in 3T3 Li cells, which have previously been shown to possess receptors for bradykinin [43]. As was the case for HeLa

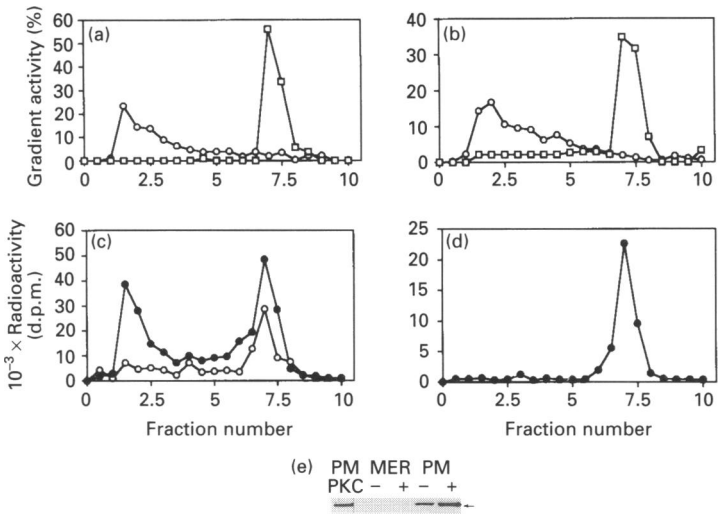

Figure 5 Distribution of PEth and PKC-α in the subcellular fractions isolated from unlabelled HeLa cells after mixing with PM fractions containing preformed [^{3}H]PEth and PKC-α

Cells prelabelled with [^{3}H]palmitic acid and incubated with 0.5% ethanol and either 0.2% DMSO or 100 nM PMA for 30 min were harvested, disrupted and fractionated on a Percoll density gradient as outlined in the Materials and methods section. Fractions were analysed for marker enzyme activity and [^{3}H]PEth accumulation as outlined below and those enriched in PM (fractions 7 and 7.5) were combined and used for either PEth or PKC-α mixing experiments. For PEth mixing experiments, the labelled PM suspension was added to unlabelled cells that had been harvested from two 150 mm dishes. The cell-membrane mixture was disrupted, fractionated on a Percoll density gradient and the gradient fractions analysed for marker enzyme activity and for [^{3}H]PEth accumulation. Rotenone-insensitive NADH–cytochrome *c* reductase activity (○), 5′-nucleotidase activity (□) (**a, b**) and [^{3}H]PEth accumulation (○, 0.2% DMSO; ●, 100 nM PMA; **c, d**) were measured for gradient fractions obtained from labelled cells (**a, c**) and unlabelled cells mixed with labelled membrane (**b, d**). For PKC-α mixing experiments, the combined PM-enriched fractions were divided into two aliquots. One was analysed directly for PM PKC-α (**e**) and the other was added to one of two recently harvested cell preparations (each from two 150 mm plates). Each cell preparation was disrupted and fractionated on Percoll density gradients. The fractions were analysed for marker enzyme activity (results not shown), and those enriched in MER and PM were analysed for PKC-α. The PKC-α associated with the MER and PM fractions isolated from both the unmixed (−) cell preparation and the cell/PM mixed preparation (+) is shown in (**e**). Marker enzyme assays, lipid analysis and Western-blot analysis were carried out as described in the Materials and methods section.

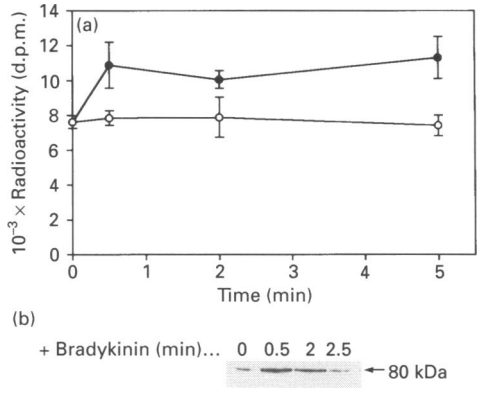

Figure 6 (a) Time course of PEth accumulation and (b) PKC-α translocation in bradykinin-stimulated 3T3 Li cells

(**a**) Cells prelabelled with [^{3}H]palmitic acid were incubated for the indicated times with 0.5% ethanol in the absence (○) or presence (●) of 1 μM bradykinin. As shown by others [44], this concentration of bradykinin was found to be optimal (results not shown). Lipids were extracted and the radioactivity associated with PEth was determined as outlined in the Materials and methods section. Each point represents the mean ± S.E.M. of three replicate determinations. (**b**) PKC-α translocation was assessed by Western-blot analysis as described in the Materials and methods section.

cells, 3T3 Li cells also predominantly expressed PKC-α with smaller amounts of PKC-ε and PKC-ζ. The accumulation of [^{3}H]PEth and translocation of PKC-α was measured in ethanol-treated 3T3 Li fibroblasts prelabelled with [^{3}H]palmitic acid. Bradykinin stimulated a rapid accumulation of [^{3}H]PEth in these cells (Figure 6a). Radioactivity associated with PEth increased to a maximum at 30 s, after which it plateaued, indicating that bradykinin caused only a transient activation of PLD. This is consistent with the effects of this agonist on PKC-α which underwent a rapid and transient association with cell membranes that peaked 30 s after cell stimulation (Figure 6b).

To identify the membrane site of PEth accumulation, subcellular fractionation of 3T3 Li cells was carried out on Percoll density gradients. The distribution of [^{3}H]PEth and PKC-α in the PM and MER membranes after bradykinin stimulation is shown in Table 2 and Figure 7 respectively. Although bradykinin

Table 2 Time course of PEth accumulation in MER and PM subcellular fractions of bradykinin-stimulated 3T3 Li cells

Cells prelabelled with [^{3}H]palmitic acid for 24 h were incubated with 1 μM bradykinin for the indicated times during a 5 min incubation with ethanol. The PM- and MER (combined MER I/II)-enriched fractions were isolated on a Percoll density gradient, and the total radioactivity associated with PEth was determined as described in the Materials and methods section. Results are expressed as means ± S.E.M. of three replicate determinations. Similar results were obtained in three separate experiments.

	Subcellular distribution of [^{3}H]PEth (total d.p.m.)	
Time (min)	MER	PM
0	3284 ± 62	4400 ± 114
0.5	4144 ± 123	4804 ± 214
2	4892 ± 602	4476 ± 356
5	5752 ± 615	5276 ± 441

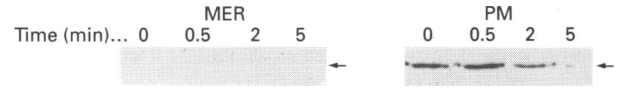

Figure 7 PKC-α translocation to the MER and PM subcellular fraction of bradykinin-stimulated 3T3 Li cells

Cells prelabelled with [^{3}H]palmitic acid for 24 h were incubated with 1 μM bradykinin for the indicated times during a 5 min incubation with ethanol. The PM- and MER (combined MER I/II)-enriched fractions were isolated on a Percoll density gradient. The effect of bradykinin on PKC-α in these fractions was assessed using Western-blot analysis as described in the Materials and methods section.

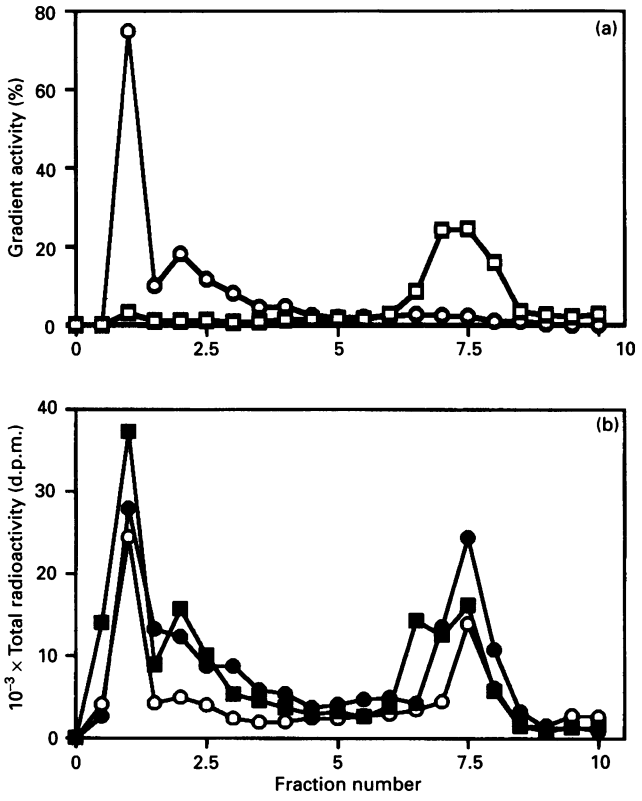

Figure 8 Distribution of PBut in subcellular fractions isolated from bradykinin-stimulated 3T3 Li cells

Cells prelabelled with [^{3}H]palmitic acid as described in the Materials and methods section were incubated with 0.5% butanol in the presence or absence of 1 μM bradykinin as indicated below. (**a**) Cells were then harvested, disrupted and fractionated on Percoll density gradients and the fractions analysed for rotenone-insensitive NADH–cytochrome *c* reductase activity (○) and 5′-nucleotidase activity (□). (**b**) Radioactivity associated with PBut was measured for gradient fractions incubated with butanol for 5 min (○), butanol for 3 min followed by a further incubation for 2 min in the presence of bradykinin (●) or butanol and bradykinin for 5 min (■). Enzyme assays and lipid analysis were carried out as described in the Materials and methods section.

appeared to increase the amount of radioactivity associated with PEth extracted from the PM, the differences were not significantly different at any time point (Table 2). In contrast, bradykinin induced a significant ($P < 0.001$) and prolonged accumulation of labelled PEth in the MER fraction. A similar result was obtained in a separate experiment in which the subcellular accumulation of PBut was measured in cells stimulated with bradykinin in the presence of butanol. Figure 8 shows the distribution of subcellular marker activity and [^{3}H]PBut in membrane fractions obtained after the fractionation of these cells on a Percoll density gradient. Whereas bradykinin stimulated a transient accumulation of [^{3}H]PBut in the PM fraction of cells (2 min), it caused a sustained accumulation of labelled PBut in the MER I and MER II fractions at all time points. Mixing experiments, similar to those carried out for the subcellular fractions of HeLa cells, indicated that the intracellular localization of both PEth and PBut occurred before cell lysis and was not due to contamination during cell disruption or centrifugation (results not shown). As shown in Figure 7, bradykinin induced a transient translocation of PKC-α to the PM, and no detectable PKC-α immunoreactivity was associated with the MER fraction. PKC-ε and PKC-ζ immunoreactivity could also not be detected in this fraction (results not shown). From these results it is therefore clear that the MER-associated accumulation of labelled PEth occurs in the absence of any detectable translocation of PKC-α to this fraction. It is possible that either bradykinin activates an MER-associated PLD by a PKC-α-independent mechanism or other forms of PKC not examined in this study are translocated to the MER fraction in response to bradykinin. However, an involvement of PKC in the bradykinin-stimulated accumulation of PEth in these cells is supported by studies in which prolonged incubation with PMA down-regulated PKC-α immunoreactivity and abolished bradykinin stimulation of PEth formation (results not shown). In addition, there is indirect evidence from other cell lines that PKC-α is involved in the regulation of PLD activity [14,29]. Consequently, as a working hypothesis we propose that PLD activation by bradykinin leads to the initial formation of PEth in the PM followed by its rapid redistribution to the MER fraction. In current studies we are examining the link between PLD activation and PKC-α using antisense technology.

Whatever the mechanism for the accumulation of PLD reaction product in the MER fraction, this study has wide-ranging implications for mechanisms of transmembrane signalling involving lipid-derived second-messenger molecules. Although agonist binding occurs in the PM, it is obvious that the metabolites that are generated by lipid hydrolysis accumulate in multiple membrane compartments. Future experiments will extend the subcellular fractionation protocol described in this paper to the study of the nucleus and other subcellular compartments that may function as sites of second-messenger accumulation.

This work was supported by grants from the Anti-Cancer Foundation of the Universities of South Australia, the National Health and Medical Research Council and the Australian Research Council.

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Received 10 November 1994/ 16 January 1995; accepted 25 January 1995