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Phospholipid asymmetry in plasma membrane vesicles derived from BHK cells

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Abstract

The transbilayer distribution of phospholipids in plasma membrane vesicles derived from BHK cells by treatment with iodoacetamide or fluoride and merocyanine 540 has been examined by exposing the vesicles to bee venom phospholipase A_2 (PLA_2) or to *Bacillus cereus* sphingomyelinase. The results show that almost all of the phosphatidylserine (PS) is on the inner lipid leaflet and most of the sphingomyelin is on the outer lipid leaflet. In contrast, about 50% of the phosphatidylcholine (PC) and 30–40% of the phosphatidylethanolamine (PE) is rapidly degraded by PLA_2 and thus appears to be present on the surface of the vesicles. The pools of PC and PE which are accessible only slowly to PLA_2 are degraded with halftimes of about 5 h and 2 h, respectively, and it is suggested that this rate reflects the rate of transbilayer migration of these lipids. We conclude that the profound energy depletion caused by treatment with iodoacetamide or fluoride does not alter the asymmetric distribution of PS across the plasma membrane but does have a marked effect on the transbilayer distribution of PE. Residual cells after treatment with fluoride and MC540 were also exposed to PLA_2. The results were broadly in agreement with those obtained with vesicles, suggesting that the vesicles were representative of the BHK cell plasma membrane in terms of phospholipid asymmetry. Fluoride or MC540 added separately caused little vesicle release but did lead to significant loss of phospholipid asymmetry. When centrifuged on a sucrose density gradient, vesicles were separated into two major fractions accounting for about two thirds and about 20%, respectively, of total phospholipid but no significant differences were seen in the transbilayer phospholipid asymmetry of the two fractions.

Key words: Phospholipid asymmetry; Plasma membrane; Vesicle; (BHK cell)

1. Introduction

Compelling evidence now exists that the phospholipids of eukaryotic plasma membranes are asymmetrically distributed between the two component lipid leaflets [1–3]. The most comprehensively studied system is that of the human erythrocyte where it has been established that phosphatidylethanolamine (PE) and phosphatidylserine (PS) are primarily located in the inner leaflet, whereas sphingomyelin, and to a lesser extent phosphatidylcholine (PC), are concentrated in the outer leaflet [4]. The phospholipid transbilayer distribution within the plasma membrane of other cell types is less well characterized largely due to the difficulty of isolating pure plasma membrane in the form of sealed vesicles. However, viruses which acquire their lipid envelope as they bud through the host cell plasma membrane have an asymmetric distribution of phospholipids not unlike that of erythrocytes [5,6], suggesting that the host cell plasma membrane must also be similar to the red cell model in terms of phospholipid asymmetry.

Over the past decade it has become clear that the long-term maintenance of phospholipid asymmetry across lipid bilayers depends on an energy-requiring process [7–9]. Initial studies on erythrocytes demonstrated an ATP-dependent translocase which specifically transports PS and PE from the outer to the inner membrane leaflet [10] and a corresponding protein has since been discovered in the plasma membranes of many other eukaryotic cell types [3]. However, the aminophospholipid translocase is probably not the only factor which maintains phospholipid asymmetry since asymmetry is maintained in energy-depleted red cells

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[11] but is gradually lost in hyperglycaemic red cells which still have an active aminophospholipid translocase [12].

Previous work in this laboratory has led to the development of a procedure for the isolation of plasma membrane exovesicles from BHK-21 cells treated with merocyanine 540 and agents which cause energy depletion [13,14]. The lipid composition of these vesicles is similar to that of red cell membranes or of enveloped virus released from BHK cells [6], showing large amounts of SM, PS and cholesterol but very small amounts of triacylglycerol and cholesterol ester. Since vesicle release is achieved relatively rapidly and without cell lysis it was thought possible that like enveloped viruses [6], the phospholipid distribution across the vesicle bilayer might reflect that of the original cell, thus providing a useful way of assessing plasma membrane lipid asymmetry. On the other hand, it might be predicted that the use of energy depletion would inactivate any ATP-dependent translocase and thus promote loss of lipid asymmetry. In order to resolve these possibilities, we decided to expose these plasma membrane vesicles to bee venom phospholipase A_2 and bacterial sphingomyelinase which have previously been used successfully to analyse phospholipid asymmetry in red cells, red cell vesicles [15], platelets [16] and in viruses [6].

2. Materials and methods

Unless otherwise stated, all reagents were obtained from Sigma, Poole, Dorset, UK.

BHK21 cells were cultured in 3.5-cm or 10-cm diameter dishes using the Glasgow modification of Minimal Essential Medium (GMEM) (ICN Flow, High Wycombe, Bucks., UK), supplemented with 5% foetal calf serum (FCS) and tryptose phosphate broth. Initial cell concentrations were such that confluency was just reached after 48 h. Cells were labelled close to equilibrium with 30 μCi/ml [^{32}P]phosphate or with 3 μCi/ml [^{35}S]methionine (Amersham) added at the beginning of the culture period.

2.1. Determination of phospholipid asymmetry

Plasma membrane vesicles were prepared from ^{32}P-labelled cells as previously described [14]. Briefly, the cells in one 10 cm dish were washed twice with phosphate-buffered saline (PBS) before incubation with 10 mM iodoacetamide in PBS at 37°C for 30 min. MC540 was then added to a final concentration of 20 μM and the incubation continued. After a further 60 min the medium was removed, centrifuged initially for 5 min at $500 \times g$ to remove any intact cells, and then at 100 000 $\times g$ for 2 h at 4°C in a swing-out rotor, to sediment the vesicles. The pellet was resuspended in 0.4 ml of 130 mM NaCl, 20 mM Hepes-NaOH (pH 7.4) (Hepes-saline). Vesicles were also prepared using 30 mM NaF instead of iodoacetamide.

15 μl of vesicle suspension was added to glass tubes held at 37°C, each containing 0.5 ml of Hepes-saline/1 mM $CaCl_2$/1 mM $MgCl_2$ containing 1.0 I.U. of bee venom phospholipase A_2 (PLA_2) or 0.2 units of *B. cereus* sphingomyelinase. At the specified times the reaction was terminated and the lipids extracted by the addition of 1.9 ml of methanol/chloroform (2:1, v/v). After phase partition of this extract by the method of Bligh and Dyer [3] and separation of phospholipids by TLC as previously described [6], the lipids were localized by autoradiography and identified by comparison with known standards. Individual spots were excised and the lipids extracted from the silica with 0.2 ml of methanol:glacial acetic acid/H_2O (5:2:3 by vol.). Each sample was counted by Cerenkov counting in 2 ml of distilled water using a Canberra Packard 2500 scintillation counter. In all experiments the efficacy of the enzyme was checked through its action on vesicles disrupted with 0.1% Triton X-100.

In some experiments, intact cells labelled to equilibrium with ^{32}P were subjected to attack by PLA_2 or sphingomyelinase. In these experiments cells were pretreated for 1 h with PBS alone or with the addition of 30 mM NaF, 20 μM MC540 or a combination of these two agents. In some experiments, 10 mM iodoacetamide was used instead of NaF. The medium was removed and 10 I.U. of PLA_2 or 1 unit of sphingomyelinase was added for 5 min or 60 min as described above for the vesicles, before extraction and analysis of phospholipids.

2.2. Density gradient subfractionation of vesicles

Plasma membrane vesicles were prepared from BHK cells labelled to equilibrium with either [^{32}P]phosphate or [^{35}S]methionine. The vesicles were resuspended in 0.5 ml of PBS and carefully layered on to prepared sucrose gradients consisting of 1.5 ml of each of 20, 25, 30, 35, 40, 45 and 50% (w/v) sucrose solutions. The gradients were then centrifuged at 100 000 $\times g$ in a Beckman SW41 rotor for 18 h at 4°C. Fractions were removed from the top of each gradient in 22 0.5-ml aliquots using an automatic pipette and the sucrose concentration in each was measured using a refractometer. The pellet was finally resuspended in 0.5 ml of PBS and analysed as fraction 23. ^{32}P-labelled samples were then extracted with chloroform/methanol and phase partitioned as above. The lower (chloro-

form) phase from each sample was dried, redissolved in 2 ml of chloroform and counted (Cerenkov radiation). In some experiments, samples of the fractionated vesicles were exposed to PLA_2 before extraction and analysis of lipids. ^{35}S-labelled samples were counted directly in scintillation fluid.

3. Results

3.1. Vesicle phospholipid asymmetry

Fig. 1 illustrates the degradation of ^{32}P-labelled phospholipids in plasma membrane vesicles treated with either phospholipase A_2 (a,b,c) or sphingomyelinase (d). 30–40% of PE, 50–55% of the PC but less than 5% of the PS were degraded within 10 min of treatment with PLA_2. 80–85% of the SM was broken down by sphingomyelinase. The remaining phospholipid underwent a very much slower degradation with halftimes of about 2 h for PE, 5 h for PC and longer for PS and SM. In the presence of Triton X-100, essentially all the phospholipid was degraded rapidly. Breakdown of PC (but not PE) was always more than the apparent production of the corresponding lysoPC. This was explained by the consistent observation that about 20% of the total lysoPC passed into the upper layer of the Bligh and Dyer phase partition in these

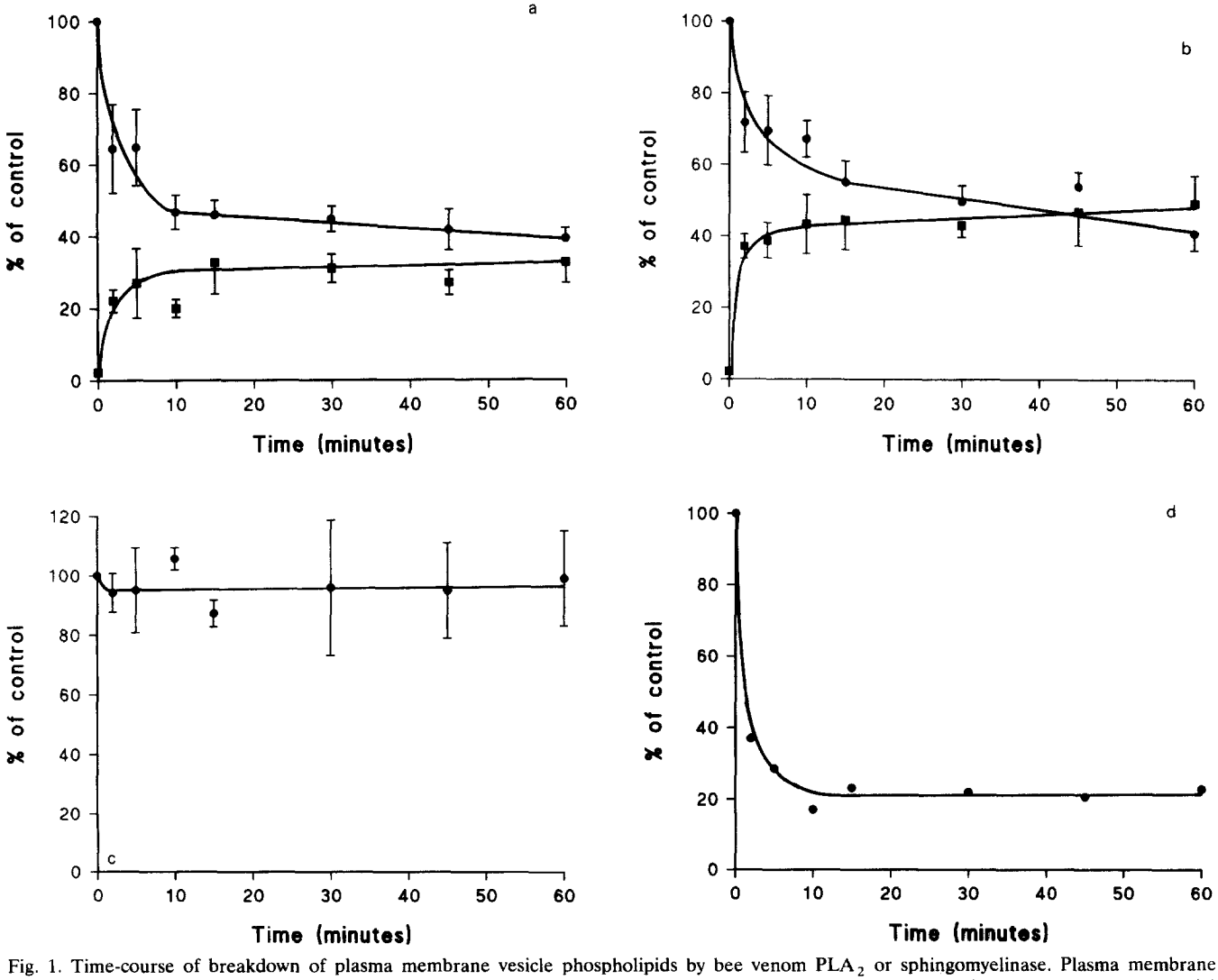

Fig. 1. Time-course of breakdown of plasma membrane vesicle phospholipids by bee venom PLA_2 or sphingomyelinase. Plasma membrane vesicles derived from BHK cells by incubation with iodoacetamide and MC540 were exposed to bee venom PLA_2 (a–c) or sphingomyelinase (d) for various times as described under Materials and methods. (a) PC (filled circles) and lysoPC (filled squares); (b) PE (filled circles) and lysoPE (filled squares); (c) PS; (d) SM. Results for the glycerolipids represent the means ± S.D. of three experiments using different samples of vesicles and are expressed relative to the amount of each lipid at the start of the incubation period. For SM, the results shown are for a representative experiment (one of three using different samples of vesicles).

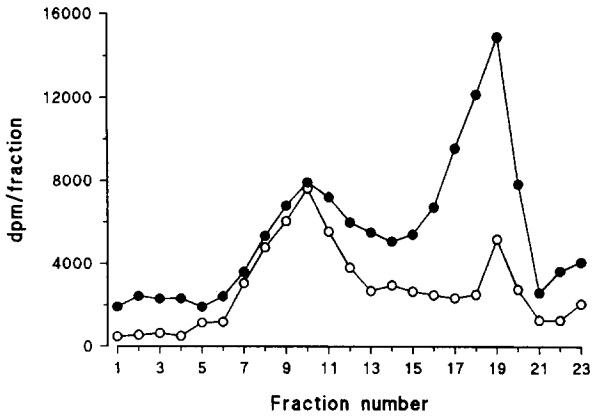

Fig. 2. Density gradient analysis of plasma membrane vesicles. Plasma membrane vesicles isolated from ^{32}P-labelled (open circles) or [^{35}S]methionine-labelled (filled circles) BHK cells treated with iodoacetamide and MC540 were centrifuged on a 20–50% w/v sucrose density gradient at 100000 × *g* for 18 h as described under Materials and methods. The pattern shown is derived from a single experiment which was repeated twice more with essentially the same result.

extractions. When vesicles were prepared using NaF to cause ATP depletion instead of iodoacetamide, yields were lower, as previously reported [14] but the pattern of lipid degradation with PLA_2 was indistinguishable from that seen with iodoacetamide vesicles (results not shown).

In order to determine if the biphasic breakdown of PE and PC could be due to heterogeneity in the composition or permeability of the vesicles, three separate preparations of vesicles prepared from cells labelled with ^{32}P or [^{35}S]methionine were subjected to analysis on sucrose density gradients centrifuged to equilibrium (Fig. 2). These experiments showed that the vesicles fell into two major density classes, a lighter one banding at a density equivalent to that of 33% (w/v) sucrose (about 1.12 g/ml) and a heavier one banding at a density equivalent to that of 46% (w/v) sucrose (about 1.17 g/ml). In terms of phospholipid radioactivity, the lighter band accounted for about two thirds of the total and the heavier band for only about 20% (Fig. 2). In contrast, the ^{35}S labelling was relatively stronger in the denser fraction suggesting that there was an approx. 3-fold greater ratio of protein to phospholipid in the denser fraction compared with the light fraction. When samples of these fractions (8–11 pooled and 18–20 pooled in Fig. 2) were exposed to PLA_2, the fractions were found to have patterns of phospholipid composition and breakdown which were indistinguishable from each other or from the original unfractionated vesicles (results not shown).

3.2. Plasma membrane phospholipid asymmetry in intact cells

Attempts were made to analyse phospholipid asymmetry in cells which had been treated with iodoacetamide with or without MC540, but no consistent results were obtained because these cells appeared to undergo lysis when treated with PLA_2, especially at longer exposure times. This problem was avoided when iodoacetamide was substituted by 30 mM fluoride which also promotes vesicle release in the presence of MC540 [14]. Labelled cells treated with fluoride, MC540 or a combination of these agents and then exposed to PLA_2 showed significant and consistent rises in concentrations of lysoPC (Fig. 3a) and lysoPE (Fig. 3b) but no measurable change in the concentration of PS or PI (results not shown). Samples from cells treated with fluoride and MC540 run on two-dimensional plates showed radioactivity in the areas of the chromatogram corresponding to LPI and LPS each equivalent to no more than 0.1% of total counts (the limit of resolution from the background in these experiments). In control

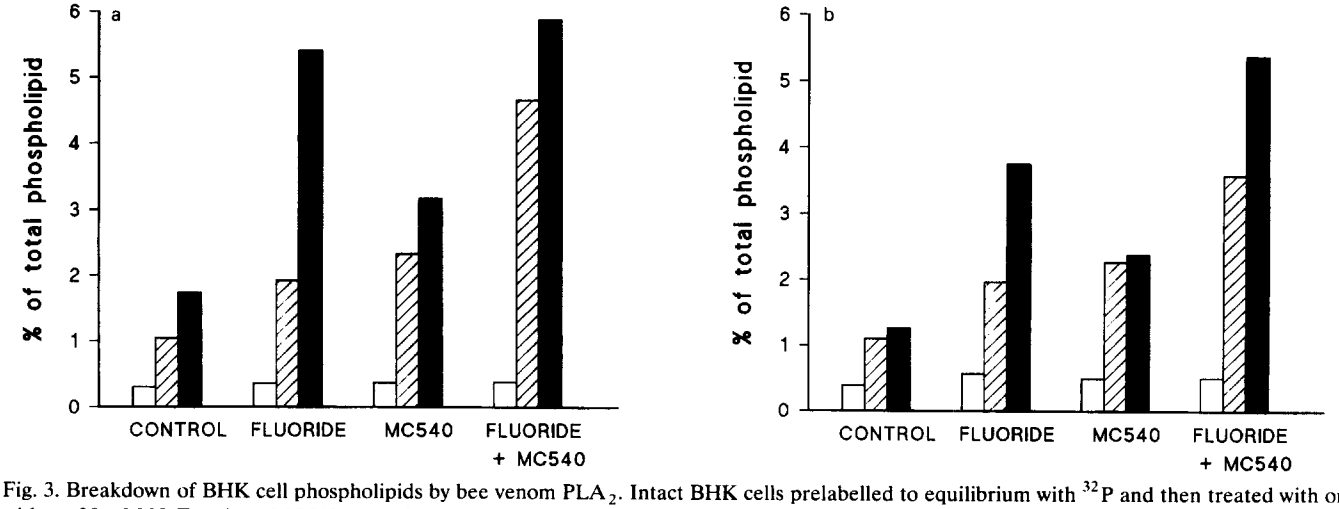

Fig. 3. Breakdown of BHK cell phospholipids by bee venom PLA_2. Intact BHK cells prelabelled to equilibrium with ^{32}P and then treated with or without 30 mM NaF and/or MC540 were either extracted immediately (open bars) or exposed to PLA_2 for 5 min (hatched bars) or 60 min (filled bars) as described under Materials and methods. Results represent the means of duplicate determinations of lysoPC (a) and lysoPE (b). The experiment was repeated on two further occasions with almost identical results.

Table 1
Breakdown of sphingomyelin by sphingomyelinase in intact cells incubated with or without fluoride

	Untreated with SMase	5 min with SMase	60 min with SMase
Control	6.71 ± 0.26	2.21 ± 0.04	1.66 ± 0.14
Fluoride	6.80 ± 0.02	2.98 ± 0.11	2.53 ± 0.14

Cells labelled to equilibrium with ^{32}P were initially incubated for 30 min with or without 30 mM NaF and MC540 as detailed under Materials and Methods. They were then washed once and then either extracted with methanol/chloroform directly or incubated for 5 min or 60 min with sphingomyelinase prior to extraction and separation of lipids. Results for cell content of sphingomyelin are expressed as means ± S.D. of triplicate determinations in one experiment which was repeated twice with very similar results.

cells incubated with or without fluoride or MC540, LPC and LPE each accounted for about 0.3% of total phospholipid. Incubation of cells for 5 min with PLA_2 in the absence of fluoride or MC540 increased LPC and LPE each by about 0.7% of total phospholipid. These values rose to about 2% of total phospholipid following preincubation with either fluoride or MC540 and to 4% of total PC and 3% of total PE after preincubation with a combination of fluoride and MC540 (Figs. 3a,b). Little further breakdown of PE occurred when incubation was continued for 60 min in control cells or in cells incubated with MC540, although a significant increase in PC breakdown was seen at 60 min. However, incubation with fluoride caused large increases (equivalent to about 4–6% of total phospholipid) in the breakdown of PE and PC when cells were incubated with PLA_2 for 60 min.

As shown previously [17], treatment of BHK cells with sphingomyelinase degrades a maximum of about 70% of total sphingomyelin and a similar finding is reproduced in Table 1 for otherwise untreated cells. In contrast, cells which had been incubated with fluoride and MC540 showed only about 85% of the sphingomyelin breakdown seen with untreated cells.

4. Discussion

The results described here demonstrate that plasma membrane vesicles derived from BHK cells maintain a considerable degree of phospholipid asymmetry, even though their preparation involves a profound depletion of cellular ATP. Thus, very little of the PS is available to PLA_2 in the intact vesicles (Fig. 1c) even though PS is readily degraded in vesicles exposed to small amounts of Triton X-100. Similarly, most of the SM is exposed at the surface and available to attack by exogenous sphingomyelinase. On the other hand, based on susceptibility to PLA_2 during a 10 min incubation, PC shows an almost equal distribution on either side of the membrane bilayer and 30–40% of the PE appears to be available at the vesicle surface. The overall pattern of phospholipid transbilayer distribution in the vesicles is rather similar to that seen in Semliki Forest virus which had obtained its lipid envelope by budding from the BHK cell surface, except that the vesicles had about twice as much PE available on the surface as the virus [6]. Assuming that the virus largely maintains the original pattern of phospholipid asymmetry present in the plasma membrane of the BHK cell, then it appears that the main change in the vesicles is a partial loss of the asymmetrical distribution of PE.

It is now well known that one of the main factors which determines plasma membrane phospholipid asymmetry is the ubiquitous presence of a translocase which moves aminophospholipids from the exoplasmic leaflet into the cytoplasmic leaflet of the bilayer [3,7–9]. This translocase requires ATP and so conditions of energy depletion might be expected to lead to collapse of the normal asymmetric distribution of aminophospholipids. The method which we have used for preparation of plasma membrane vesicles from nucleated cells depends on extreme energy depletion using either iodoacetamide or fluoride [14] and yet the vesicles still exhibit almost complete asymmetry of distribution of PS (Fig. 1c). This is a similar result to that seen with vesicles released from red cells by treatment with Ca^{2+} and ionophore A23187 which are also energy depleted [15]. The same lack of effect of energy depletion on transbilayer phospholipid asymmetry has also been demonstrated recently for intact red cells stored for long periods [11]. Thus it appears that there is little tendency for PS to undergo spontaneous transbilayer movement even under conditions of severe energy depletion. However, it is known that the affinity of the aminophospholipid translocase is greater for PS than for PE and this may explain why the orientation of PS is relatively more stable [11]. The situation is similar for SM (Fig. 1d) although breakdown with sphingomyelinase was only about 80% (less than was seen with virus budding from BHK cells 6) and this may indicate some loss of the asymmetric distribution for SM. This is also suggested by the decreased breakdown of SM in intact cells treated with fluoride and MC540 before exposure to sphingomyelinase (Table 1).

There is evidence that substitution of red cell sulphydryl groups with diamide does increase the exposure of PS to exogenous PLA_2, but to a lesser extent than for PE [18]. However, it should be noted that unlike iodoacetamide, diamide would cause protein crosslinking, which may have additional consequences for phospholipid asymmetry. It has been shown that a prolonged rise in internal Ca^{2+} concentration in erythrocytes leads to increased exposure of PS at the cell surface and this has been explained in terms of a Ca^{2+}-dependent degradation of skeletal proteins which might normally help to maintain PS on the inner lipid

leaflet of the bilayer [19]. Possibly a similar effect on the integrity of cytoskeletal proteins could explain the dramatic loss of phospholipid asymmetry in red cells under conditions of prolonged hyperglycaemia [12]. However, recent work suggests that a general phospholipid scrambling is induced by Ca^{2+} [20] and this would be difficult to ascribe to a specific effect on PS-binding proteins.

In contrast to the behaviour of PS in plasma membrane vesicles, dissipation of the transbilayer gradient of PE does appear to occur on energy depletion since there is much more of this lipid which is accessible to PLA_2 in the vesicles than in the virus envelope (Figs. 1b and 6). Furthermore, the pool of PE which is not immediately accessible to PLA_2 is degraded relatively quickly (halftime about 2 h), and faster than the equivalent pool of PC (about 5–6 h) (Fig. 1a). If this PE pool is originally on the cytoplasmic face of the vesicle membrane then the rate at which it is attacked by external PLA_2 should reflect the rate of transbilayer migration of PE. The other possibility, that the slow breakdown is due to progressive lysis of the vesicles seems unlikely because there is no slow breakdown of PS. If transbilayer migration of PE has a halftime of as little as 2 h then it would be expected that during the preparation of vesicles under conditions of energy depletion for more than 1 h, up to 25% of the PE could appear in the outer leaflet. This could explain why in freshly-prepared vesicles PE is the only phospholipid to be more susceptible to PLA_2 than it is in the virus envelope.

For PC there is no evidence in either the vesicles (Fig. 1) or the viral envelope [6] for any significant degree of asymmetrical distribution. However, the degradation of the internal pool in both cases is slow (halftime about 5 h) and is consistent with values obtained previously for the rate of transbilayer migration of PC in red cell membranes (see Ref. [7]).

Analysis of plasma membrane vesicles on a sucrose density gradient showed that there were two main density peaks which differed in their ratio of protein to phospholipid (Fig. 2). Some heterogeneity in density distribution would be consistent with electron micrographs of the vesicles which show a mixture of small (50–100 nm) vesicles together with some larger components [14]. It would generally be expected that larger buds from the cells would contain more occluded cytosolic protein and hence should be denser; this could explain the biphasic distribution of vesicles on the gradient and the relatively larger amount of radioactive methionine in the denser fraction. However, no difference was observed between the two major fractions either in terms of phospholipid composition or in the relative breakdown of phospholipids seen with PLA_2, suggesting that the difference between the fractions was probably one of vesicular size alone.

These observations introduce some complexities into the interpretation of the data on phospholipid asymmetry since not only do vesicles as small as 50 nm have significantly more phospholipid in the outer leaflet than is present in the inner leaflet (due to their high curvature) but they may bind more MC540 [21], resulting in an increased negative charge which could influence the activity of phospholipase A_2 [22]. At present it is not possible to quantify these factors.

How do these results for vesicles relate to the comparable experiments in which the residual cells after various treatments were exposed to PLA_2? Such experiments are complicated by the fact that because the outer lipid leaflet of the plasma membrane accounts for only a few per cent of the total cellular phospholipid, the results could be clouded by even a very low level of cell breakage. Results with cells exposed to iodoacetamide and MC540 were difficult to interpret because the treatment with PLA_2 caused lysis even after 5 min. However, when fluoride was substituted for iodoacetamide no measurable breakdown of PS or PI was seen in cells treated with PLA_2 for 60 min and this argues convincingly that with fluoride, cell lysis was not a problem under these conditions.

In the presence of fluoride and MC540 (conditions where vesicles are released) about 4% of total PC and 3% of PE was degraded in whole cells exposed to PLA_2 for 5 min as judged from appearance of the respective lysolipids (Fig. 3). Assuming that plasma membrane constitutes about 25% of total BHK cell phospholipid [14] and about 25% and 30%, respectively, of plasma membrane phospholipid is PE or PC, then plasma membrane accounts for about 6% of total cell PE and 8% of total PC. Thus about half of the plasma membrane content of PE and PC is degradable in 5 min when whole cells treated with fluoride and MC540 are exposed to PLA_2. This is quite consistent with the results obtained with vesicles (Figs. 1a,b) which indicate that under these conditions, there is little asymmetry in the distribution of PE and PC.

Treatment of these cells for 60 min with PLA_2 gave a breakdown of PE and PC which was equivalent to about 70% of total plasma membrane PC or 85% of plasma membrane PE (Figs. 3a,b). This is significantly greater than the equivalent values for vesicles (60%, Figs. 1a,b) and may reflect an enhanced transfer of these lipids to the surface from internal sources, perhaps mediated by cytosolic phospholipid transfer proteins [23].

In cells incubated with PBS alone, PLA_2 caused the breakdown of about 0.7% of PC and PE after 5 min (Fig. 3), suggesting that in the control cells only about 10% of the plasma membrane content of these lipids is on the outer leaflet of the bilayer. This value scarcely increased for PE when the incubation with PLA_2 was extended to 1 h but approximately doubled for PC.

These results indicate that when cells are not energy-depleted there is little tendency for PE to lose its asymmetrical distribution in favour of the inner leaflet. However, the results for PC are anomalous since they suggest a degree of lipid asymmetry for this lipid which is not seen at all in either vesicles or in virus budding from BHK cells [6]. The reason for this is not clear but could reflect a relative lack of access of the PLA_2 to PC in the control cells which is not related to the transbilayer distribution of this lipid.

Incubation of cells with either fluoride or MC540 alone caused release of only small amounts of vesicles compared with the combination of these two agents [14]. Nevertheless, either treatment caused an increase in breakdown of PE and PC at 5 min which was equivalent to about 2% of total cell content of these lipids or about 25–30% of the plasma membrane content. While it is not surprising that energy depletion with fluoride should cause an apparent loss of phospholipid asymmetry, it is not understood why MC540 alone causes a substantially similar effect even though it has no significant effect on ATP levels [14]. The possibility exists that changes in local membrane curvature [24] or in the lipid packing pressure induced by MC540 [21] could affect the orientation of membrane phospholipids or their accessibility to PLA_2. In this context it is interesting to note that vesicles derived from red cells without the use of MC540 are also much more susceptible to PLA_2 than untreated cells [15].

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