Biochimica et Biophysica Acta, 987 (1989) 199–204
Elsevier

BBAMEM 74677

Membrane phospholipid asymmetry in Semliki Forest virus grown in BHK cells

David Allan and Paul Quinn

Department of Physiology, University College and Middlesex Medical School, London (U.K.)

(Received 2 June 1989)

Key words: Phospholipid asymmetry; Semliki Forest virus; Phospholipase A_2

The distribution of phospholipids across the membrane bilayer of Semliki Forest virus grown in BHK cells has been examined by treating the virus with bee venom phospholipase A_2 and sphingomyelinase C from *Staphylococcus aureus*. From the amounts of different phospholipids which are degraded rapidly (half-time about 1 min for phospholipase A_2) we calculate that in virus isolated 16 h after infection about 95% of sphingomyelin, 55% of phosphatidylcholine, 20% of phosphatidylethanolamine and less then 5% of phosphatidylserine is present on the outer leaflet of the virus envelope. Less than 5% of the virus was permeable to macromolecules before or after treatment with phospholipases as judged by accessibility of the genome to external ribonuclease. A much slower (half-time about 1 h) breakdown by phospholipase A_2 of originally inaccessible phosphatidylcholine and phosphatidylethanolamine appeared to be due to an enzyme-induced loss of lipid asymmetry since the original asymmetric distribution of phospholipids was maintained for several hours when the virus alone was incubated at 37°C. However, virus incubated for 20 h at 37°C showed a marked loss of phosphatidylethanolamine and phosphatidylserine asymmetry and a greater susceptibility to lysis by longer treatment with phospholipase A_2.

Introduction

Considerable evidence exists that cell membranes show an asymmetric distribution of phospholipids between the two leaflets of the lipid bilayer [1]. This evidence is particularly convincing for the plasma membranes of mammalian cells, which generally have most of their sphingomyelin on the outer leaflet of the bilayer and most of their aminophospholipids on the inner leaflet, although phosphatidylcholine appears to be more evenly distributed. For the mammalian erythrocyte, which provides the classic example of phospholipid asymmetry, it has been strongly argued that this asymmetry is maintained either through interactions between aminophospholipids and protein components of the membrane skeleton [2,3] or through the agency of an ATP-dependent translocase which actively moves aminophospholipids from the outer to the inner leaflet [4–7].

Comparable evidence for lipid asymmetry in other cell types is hard to obtain because of the difficulty in isolating sufficiently pure plasma membrane samples in the form of sealed vesicles. However, one approach to this problem utilises the properties of certain viruses which acquire their membrane envelope as they bud from the surface of the host cell. These viruses appear to have a phospholipid composition which is an accurate reflection of that of the host cell plasma membrane [8–13] and it is possible that that they also conserve (at least initially) the original lipid asymmetry of the host membrane. Phospholipid asymmetry has been previously demonstrated in enveloped viruses but the published results are confusing in some respects, particularly regarding the disposition of sphingomyelin and phosphatidylserine. Early work on vesicular stomatitis virus [14] showed a pattern of phospholipid asymmetry similar to that of red cell membranes but did not include suitable controls for lysed virus and employed a phospholipase C which does not normally attack phosphatidylserine. Tsai and Lenard [15] originally demonstrated a similar pattern of phospholipid asymmetry in influenza virus but later they [16] claimed that these results were erroneous and that most of the sphingomyelin of the virus was on the inner lipid leaflet. These latter results were supported by Van Meer et al. [17] who estimated that only 33% of the sphingomyelin of Semliki Forest virus (SFV) was on the outer leaflet,

Correspondence: D. Allan, Department of Physiology, University College and Middlesex Medical School, University Street, London WC1 6JJ, U.K.

and that consequently phosphatidylserine (for which they provided no direct experimental evidence). would have to be assigned completely to the outer lipid leaflet. This conclusion would imply either that the surface membrane of BKH cells (from which the virus budded) exhibited a pattern of phospholipid asymmetry which was opposite to that of most other cells including erythrocytes [1] or that an inversion of normal orientation of the membrane occurred during viral release.

We have been particularly interested in the transmembrane disposition of sphingomyelin and phosphatidylserine as a consequence of our previous work on erythrocytes [18,19] and BHK cells [20], the results of which generally confirmed that sphingomyelin was largely confined to the outer leaflet of the plasma membrane. In an attempt to resolve some of the past inconsistencies we decided to re-examine the question of phospholipid asymmetry in SFV grown in BHK cells, with the presumption that results from the virus might also give information regarding phospholipid asymmetry in the plasma membrane of the host cell at the time of viral release.

Methods

SFV was isolated from cells grown almost to confluence as previously described [21] but with the addition of ^{32}P (50 μCi/ml) to label phospholipids or of [^{3}H]uridine (100 μCi/ml) to label RNA. Radiochemicals were obtained from Amersham International plc. Cells were infected with SFV at a multiplicity of 0.1% and virus was harvested 16 h later and purified as described previously [21]. The virus was finally resuspended at a concentration of about 50 nmol of lipid phosphorus in 0.1 ml of 130 mM NaCl, 20 mM Hepes-NaOH(pH 7.4) (Hepes-saline).

Phospholipase A_2 from bee venom (760 I.U./mg protein), sphingomyelinase from *Staphylococcus aureus* (200 I.U./mg protein) and bovine pancreatic ribonuclease (100 I.U./mg protein) were obtained from Sigma Chemical Co. 1.0 I.U. of phospholipase A_2 or 0.2 I.U. of sphingomyelinase were added to glass test tubes containing 0.5 ml of Hepes-saline, 1 mM $CaCl_2$, 1 mM $MgCl_2$ at 37°C. 10 μl of virus suspension was added to each tube and after various periods of incubation lipids were extracted by addition of 1.9 ml of methanol/chloroform (2:1, v/v). Carrier red cell lipids (0.1 μmol of phosphorus) were added and phospholipids were separated by tlc as described previously [22,23]. Individual ^{32}P-labelled spots were localised by autoradiography on Fuji X-ray film and identified by comparison with standard phospholipids (Sigma). Spots were excised, digested in 0.2 ml of 70% perchloric acid at 180°C and counted in 10 ml of water (Cerenkov radiation) using a Searle scintillation counter.

In some experiments purified virus was incubated in normal cell growth medium at 37°C for a further 20 h before treatment with phospholipases as above. Virus frozen in liquid nitrogen and then thawed was also tested with the phospholipases to see if freezing altered the disposition of phospholipids. In other experiments the integrity of the viral membrane was assessed by incubating [^{3}H]uridine-labelled virus with ribonuclease before and after treatment with phospholipases as described above. Disruption of the viral membrane with 0.5% Triton X-100 allowed complete degradation of viral RNA by the ribonuclease. Following digestion of available RNA with 0.1 mg/ml ribonuclease for 3 min at 37°C, 25 μl samples were spotted on to 3MM filter papers which were washed with four changes of 200 ml of 10% trichloroacetic acid, followed by ethanol and then ether. After air drying, the filters were counted in 10 ml PCS scintillation fluid (Amersham).

Results

Table I shows the phospholipid composition of BHK cells and the distribution of radioactivity in the phospholipids of purified SFV grown in these cells labelled to equilibrium with [^{32}P] phosphate. The pattern of labelling resembles that previously described for ^{32}P-labelled SFV and for mass distribution among the phospholipids [9,17] except that we found less phosphatidylcholine and correspondingly more sphingomyelin in the ^{32}P-labelled samples.

The breakdown of phospholipids in fresh ^{32}P-labelled virus after treatment with phospholipase A_2 (a, c) or sphingomyelinase (e) is shown in Fig. 1. Sphingomyelinase caused the loss of more than 95% of total sphingomyelin radioactivity without any measureable effect on other phospholipids and with no significant increase in susceptibility of viral RNA to ribonuclease (Table II). The simplest interpretation of this result is

TABLE I

The phospholipid composition of unlabelled BHK cells and the distribution of radioactivity among the phospholipids of SFV isolated from BHK cells labelled to equilibrium with ^{32}P

Phospholipids were extracted and analysed as described under Methods. Results are expressed as percentage of total phospholipid phosphorus (cells) or radioactivity (virus) and represent the means from five experiments with unlabelled cells and five experiments with ^{32}P-labelled virus.

	..lls	^{32}P virus
Phosphatidate	2.1 ± 1.0	3.2 ± 1.2
Phosphatidylethanolamine	24.0 ± 1.3	28.1 ± 1.6
Phosphatidylinositol	8.7 ± 1.3	1.0 ± 0.3
Phosphatidylserine	9.0 ± 0.9	17.6 ± 1.1
Lysophosphatidylethanolamine	< 0.5	0.5 ± 0.2
Phosphatidylcholine	46.1 ± 2.2	21.8 ± 0.7
Sphingomyelin	8.9 ± 0.3	26.6 ± 2.6
Lysophosphatidylcholine	< 0.5	1.1 ± 0.4

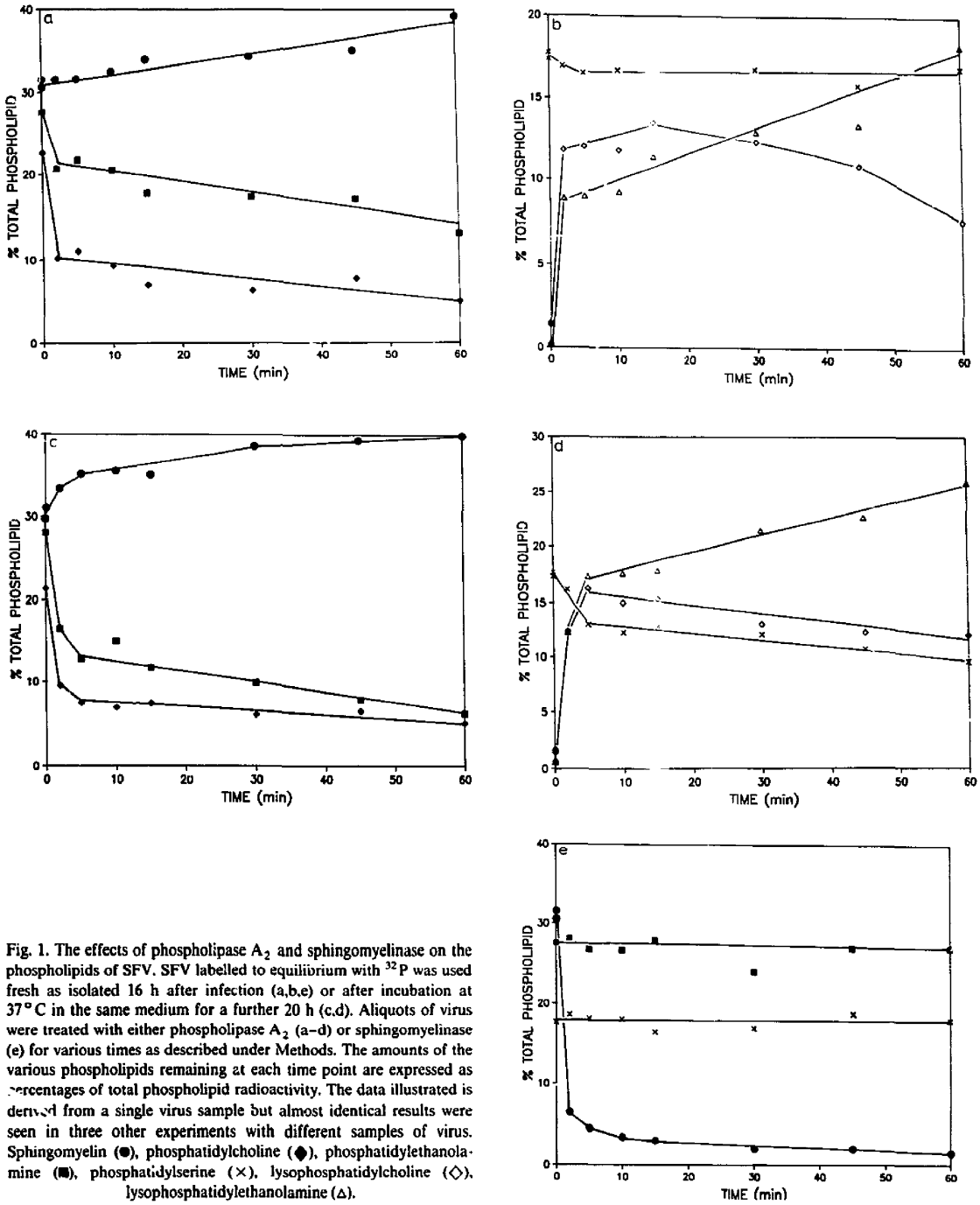

Fig. 1. The effects of phospholipase A_2 and sphingomyelinase on the phospholipids of SFV. SFV labelled to equilibrium with ^{32}P was used fresh as isolated 16 h after infection (a,b,e) or after incubation at 37°C in the same medium for a further 20 h (c,d). Aliquots of virus were treated with either phospholipase A_2 (a–d) or sphingomyelinase (e) for various times as described under Methods. The amounts of the various phospholipids remaining at each time point are expressed as percentages of total phospholipid radioactivity. The data illustrated is derived from a single virus sample but almost identical results were seen in three other experiments with different samples of virus. Sphingomyelin (●), phosphatidylcholine (◆), phosphatidylethanolamine (■), phosphatidylserine (×), lysophosphatidylcholine (◇), lysophosphatidylethanolamine (△).

that essentially all the sphingomyelin of the virus is available on the outer leaflet of the bilayer. Exactly the opposite conclusion was reached regarding the disposition of phosphatidylserine, since exposure of virus to phospholipase A_2 caused less than 5% breakdown of this phospholipid even after incubation for 1 h (Fig. 1b). Most of this breakdown occurred quickly and could largely be explained by the presence of a small amount

TABLE II

The susceptibility of fresh and aged SFV to attack by phospholipases and ribonuclease

SFV labelled with either ^{32}P or with [^{3}H]uridine was incubated with phospholipase A_2 (2 I.U./ml), sphingomyelinase (0.4 I.U./ml) and/or with ribonuclease (0.1 mg/ml) as indicated below. Breakdown of viral phospholipids and RNA was measured as described under Methods. Virus was used either fresh (as isolated 16 h after infection) (a) or after an additional 20 h incubation in growth medium (b). Results are presented as means ± S.D. from four experiments.

	% phospholipid breakdown by phospholipase A_2 (3 min) or * sphingomyelinase (30 min)	
	(a)	(b)
PE	23 ± 3	54 ± 4
PS	6 ± 2	30 ± 5
PC	54 ± 4	68 ± 5
SM *	95 ± 3	97 ± 6
	% RNA breakdown by ribonuclease (3 min) after various pretreatments	
	(a)	(b)
Untreated	3 ± 2	0
Phospholipase A_2 (3 min)	5 ± 3	7 ± 4
Phospholipase A_2 (30 min)	7 ± 3	71 ± 6
Sphingomyelinase (30 min)	4 ± 2	6 ± 3
Triton X-100 (0.1%)	99 ± 3	98 ± 2

of lysed virus since about 5% of the total viral RNA was rapidly available to ribonuclease (Table II). Similar results were seen for phosphatidylinositol although the data (not shown) were less convincing due to the low levels of this phospholipid. The glycerophospholipids were almost completely degraded by phospholipase A_2 when the virus was lysed by addition of 0.5% Triton X-100 (not shown).

Other phospholipids showed susceptibilities to phospholipase A_2 which were intermediate between the extreme cases represented by sphingomyelin and phosphatidylserine (Fig. 1a). Thus about 55% of phosphatidylcholine was degraded rapidly (half time about 1 min) whereas the remainder was broken down much more slowly (half-time about 1 h). With phosphatidylethanolamine, about 20% disappeared with a half-time of about a minute while the remainder was degraded slowly (half-time about 1.5 h). There was an increase in lysophosphatidylethanolamine reciprocal to the drop in phosphatidylethanolamine, but especially at later time points there appeared to be insufficient lysophosphatidylcholine to account for the decrease in phosphatidylcholine, presumably because this lysolipid was subject to further degradation by an enzyme activity contaminating the phospholipase A_2 giving rise to water-soluble radioactivity. This would explain the apparent increase in sphingomyelin as a proportion of total lipid during prolonged incubation with phospholipase A_2 and suggests that the proportions of the other phospholipids may be similarly overestimated at longer incubation times.

Only a small proportion of the lipid which was degraded by phospholipase A_2 in 3 min could be ascribed to the presence of lysed virions; based on the susceptibility of RNA in [^{3}H]uridine-labelled virus to added ribonuclease it was estimated that only about 5% of the virus was lysed (Table II). Even treatment for 30 min with phospholipase A_2 or sphingomyelinase caused little increase in the permeability of the viral membrane to macromolecules as judged by the failure of added ribonuclease to attack more viral RNA (Table II). This suggests that phospholipase A_2 is unable to penetrate the viral membrane and that accordingly, pools of phospholipid which are degraded rapidly (within 3 min) by this enzyme are likely to be present in the outer leaflet of the membrane bilayer. On this basis, the transbilayer distribution of phospholipids in the viral membrane is as shown in Table III, with essentially all the sphingomyelin together with 55% of phosphatidylcholine and 20% of phosphatidylethanolamine present in the outer leaflet and all the remaining phospholipid in the inner leaflet.

The slow degradation of phosphatidylcholine and phosphatidylethanolamine which occurred after the initial fast breakdown was not associated with increased viral lysis as measured by susceptibility to ribonuclease (Table II). Neither did it seem to reflect a time-dependent loss of phospholipid asymmetry in the viral membrane since virus preincubated for two hours at 37°C before addition of phospholipase A_2 showed a breakdown of phospholipids which was indistinguishable from unincubated virus (not shown). Only after preincubation for 20 h at 37°C was there a pronounced increase in immediate susceptibility of phosphatidylethanolamine (and to a lesser extent phosphatidylserine) to phospholiphase A_2 (Fig. 1, Table II). After 3 min exposure to phospholipase A_2, more than 50% of phosphatidylethanolamine and 30% of phosphatidylserine was degraded together with about two thirds of the phosphatidylcholine. A larger apparent increase in the proportion of sphingomyelin in this experiment was due

TABLE III

The distribution of phospholipids across the plasma membrane of SFV

Results are expressed as moles/100 moles of total phospholipid.

	Inner leaflet	Outer leaflet
PA	3	0
PE	22	6
PS	17	0
PI	1	0
PC	9	13
SM	1	26
Total	53	45

partly to the progressive loss of lysophosphatidylcholine referred to above and partly to the fact that lysophosphatidylserine chromatographed in the same region as sphingomyelin. Sphingomyelin degradation by sphingomyelinase was > 90%. The phospholipid breakdown could not be explained by an increase in viral lysis as judged by susceptibility of viral RNA to ribonuclease (Table II); the implication is that the viral membrane has lost its original asymmetry. However, this preincubated virus was much more susceptible to lysis by longer treatment with phospholipase A_2 since after 30 min with this enzyme, over 70% of viral RNA was available to ribonuclease (Table II). In all the above experiments virus which had been stored overnight 4°C or frozen and then thawed, behaved indistinguishably from fresh virus, although repeated freeze-thawing did lead to increases in susceptibility to ribonuclease and phospholipase A_2.

Discussion

The distribution of radioactivity in the phospholipids of SFV reported here (Fig. 1., Table I) is very similar to that described previously [9,17] although our results show rather more sphingomyelin and less phosphatidylcholine than those of other workers. These deviations did not reflect differences in the overall composition of the BHK cells which closely resembled that described in previous reports (Table I). Since the lipids of an enveloped virus are thought to be a representative sample of those of the host cell membrane from which the virus buds [8–13], the viral lipid composition should also represent the lipid composition of the BHK cell plasma membrane at the time of viral release. Based on a comparison of the composition of intact BHK cells (Table I) with that of isolated virus and assuming that 65% of total sphingomyelin is on the cell surface [20] it can be calculated that 21–22% of total cell phospholipid resides in the plasma membrane. This figure is considerably higher than a value for the relative area of plasma membrane based on morphometry of BHK cell [24] but recent evidence suggests that morphometric techniques have systematically underestimated plasma membrane area. Revised analyses now suggest a value close to 20% (Griffiths, G., personal communication).

It is interesting to note that the viral phospholipid composition (and thus that of the BHK cell plasma membrane) resembles the composition of human erythrocytes and myelin [25] which represent the two purest samples of plasma membrane from other cell types. This suggests that even in very distantly related cells the phospholipid composition of plasma membranes is rather similar and distinct from that of intracellular membranes. In particular, plasma membranes appear to possess relatively large amounts of sphingomyelin and phosphatidylserine compared to intracellular membranes and relatively small amounts of phosphatidylcholine and phosphatidylinositol. There are many reported phospholipid compositions of plasma membranes isolated from a variety of different cell which do not conform to the paradigm suggested by results such as ours; in these cases we would emphasise the well-known difficulties involved in isolating a pure sample of plasma membrane from complex cells by conventional procedures of subcellular fractionation.

The virus and thus by implication, the plasma membrane of the BHK cell, also resembles the erythrocyte plasma membrane [1] in terms of the asymmetric distribution of phospholipids with most of the sphingomyelin located externally and most of the anionic phospholipid residing in the inner leaflet of the bilayer (Table III). However, phosphatidylcholine was distributed more symmetrically in the viral membrane than in the human erythrocyte. Assuming that glycolipid accounts for about 8.6 mol/100 mol of phospholipid [9] and that it is present largely in the outer lipid leaflet, it can be seen that as expected, approximately equal amounts of polar lipids are present in each leaflet of the viral membrane.

It is not clear why some previous workers have concluded that unlike the erythrocyte, the virus has a substantial fraction of its sphingomyelin on the inner lipid leaflet and most of its phosphatidylserine on the outer leaflet. It may be significant that Lenard and co-workers [15,26], using influenza virus, employed a phospholipase C from *Clostridium welchii* which does not attack sphingomyelin specifically and a phospholipid transfer protein with no convincing ability to transfer sphingomyelin [27]. Van Meer et al. [17] used the specific *S. aureus* sphingomyelinase (which seems to require Mg^{2+} for activity) but in the presence of 10 mM Ca^{2+} at suboptimal temperatures. Although the latter workers did observed complete breakdown of viral sphingomyelin (at 37°C and with 100 times as much enzyme as we have used) they interpreted this as a lytic artefact. This was not the case in our experiments since susceptibility of viral RNA to RNAase remained low. There is a formal possibility that sphingomyelinase can induce migration of putative inner leaflet sphingomyelin to the surface but there is no previous evidence for such an effect of this enzyme and we saw no indication of the biphasic kinetics which might be expected if there were two distinct pools of sphingomyelin. Using the simplest conclusion that essentially all the sphingomyelin is originally on the surface, we can assign a bilayer localisation for > 95% of the total lipid (Table III) unlike some other workers [15–17,26] who could not assign any more than 80% of polar lipids to either inner or outer leaflet.

In contrast with the data on sphingomyelin, our results showing about 55% of phosphatidylcholine and 20% of phosphatidylethanolamine exposed on the surface of the virus agree well with previous work

[15–17]. However it is not clear what is the cause of the slow development of accessibility to phospholipase A_2 after the initial fast breakdown of the surface fraction of these phospholipids. A comparable slow phase of accessibility to either phospholipase C or TNBS was observed by van Meer et al. [17]. This observation is not due to viral lysis since as noted above, there was no increased exposure of RNA and phosphatidylserine showed no increased susceptibility to phospholipase A_2. The most likely explanation is that treatment with phospholipase or TNBS disrupts the normally stable asymmetric distribution of phosphatidylcholine and phosphatidylethanolamine, allowing the internal pools to come to the surface much more rapidly than usual. A similar mechanism has been proposed by Frank et al. [28] to account for certain anomalous features of the attack of phospholipase A_2 on red cells.

Interestingly, the transbilayer distribution of phosphatidylserine seems to be extremely stable, even when phosphatidylcholine and phosphatidylethanolamine organisation has been disturbed by phospholipase A_2 attack. Only after incubation at 37°C for 20 h were large amounts of phosphatidylserine readily available to external phospholipase A_2 and under these conditions half of the phosphatidylethanolamine was available to the enzyme. This occurred in the absence of viral lysis as judged by susceptibility to ribonuclease and suggests that our virus preparation had virtually lost the original asymmetric distribution of anionic lipids. On the other hand, availability of sphingomyelin to sphingomyelinase was scarcely altered and breakdown of phosphatidylcholine by phospholipase A_2 was not greatly increased by preincubation of virus at 37°C. The results imply that in SFV, asymmetry of distribution is lost most quickly by phosphatidylethanolamine (half-time < 10 h), followed by phosphatidylserine (half-time > 20 h) and phosphatidylcholine (half-time > 40 h). No evidence for an alteration in the asymmetric distribution of sphingomyelin was observed. These results are not inconsistent with previous data which suggests a very slow rate of transbilayer migration for phosphatidylcholine and especially for sphingomyelin [26,27].

It has been suggested previously that in the red cell membrane, phosphatidylserine and phosphatidylethanolamine are generally confined to the inner leaflet due to an energy-dependent phospholipid transport process [4–6] and/or to specific interaction of phosphatidylserine with the skeletal protein, spectrin [2,3]. Viruses do not possess either skeletal proteins or an energy source, so that perhaps it is not surprising that we see the gradual exposure of phosphatidylethanolamine and phosphatidylserine on the surface of the virus after prolonged incubation at 37°C. Similar conclusions were reached in our previous studies of phospholipid asymmetry in spectrin-free microvesicles derived from normal red cells [29] and in free spicules isolated from sickle cells [30]. However, it might be misleading to extrapolate from these results to metabolically active membranes such as those of BHK cells which may possess specific proteins to accelerate transbilayer migration of phospholipids and thus would need an active process to maintain phospholipid asymmetry.

Acknowledgements

We thank the Medical Research Council and the Sir Halley Stewart Trust for their support.

References

1 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71.
2 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352.
3 Kumar, A. and Gupta, C.M. (1983) Nature (London) 303, 632–633.
4 Zackowski, A., Fellmann, B. and Devaux, P.F. (1985) Biochim. Biophys. Acta 815, 510–514.
5 Bitbol, M. and Devaux, P.F. (1988) Proc. Natl. Acad. Sci. USA 85, 6783–6787.
6 Middelkoop, E., Coppens, A., Llanillo, M., Van der Hoek, E.E., Slotboom, A.J., Lubin, B.H., Op den Kamp, J.A.F., Van Deenen, L.L.M. and Roloefsen, B. (1989) Biochim. Biophys. Acta 978, 241–248.
7 Daleke, D.L. and Huestis, W.H. (1989) J. Cell Biol. 108, 1375–1385.
8 Klenk, H.-D. and Choppin, P.W. (1969) Virology 38, 255–268.
9 Renkonen, O., Kääränen, L., Simons, K. and Gahmberg, G. (1971) Virology, 46, 318–326.
10 McSharry, J.J. and Wagner, R.R. (1971) J. Virol, 7, 59–70.
11 Quigley, J.P., Rifkin, D.B. and Reich, E. (1971) Virology 46, 106–116.
12 Hirschberg, C.B. and Robbins, P.W. (1974) Virology 61, 602–608.
13 Van Meer, G. and Simons, K. (1982) EMBO J. 1, 847–852.
14 Patzer, E.J., Moore, N.F., Barenholz, Y., Shaw, J.M. and Wagner, R.R. (1978) J. Biol. Chem. 253, 4544–4550.
15 Tsai, K.-H. and Lenard, J. (1975) Nature (London) 253, 554–555.
16 Rothman, J.E., Tsai, D.K., Dawidowicz, A. and Lenard, J. (1976) Biochemistry 15, 2361–2370.
17 Van Meer, G., Simons, K., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1981) Biochemistry 20, 1974–1981.
18 Allan, D. and Raval, P.J. (1987) Biochim. Biophys. Acta 897, 355–363.
19 Allan, D. and Walklin, C.M. (1988) Biochim. Biophys. Acta 938, 402–410.
20 Allan, D. and Quinn, P. (1988) Biochem. J. 254, 765–771.
21 Green, J., Griffiths, G., Quinn P., Lo ward, D. and Warren, G. (1981) J. Mol. Biol., 152, 663–698.
22 Skipski, V.P., Peterson, R.F. and Barclay, M. (1964) Biochem. J. 90, 374–378.
23 Allan, D. and Cockcroft, S. (1982) J. Lipid Res. 23, 1373–1374.
24 Griffiths, G., Warren, G., Quinn, P.S., Mathieu-Costello, O. and Hoppeler, H. (1984) J. Cell Biol. 98, 2133–2141.
25 Rouser, G., Nelson, G.J., Fleischer, S. and Simon, G. (1968) in Biological Membranes (Chapman, D. ed.), pp. 5–69, Academic Press, New York.
26 Lenard, J. and Rothman, J.E. (1976) Proc. Natl. Acad. Sci USA 73, 391–395.
27 Shaw, J.M., Moore, N.F., Patzer, E.J., Correa-Freire, M.C., Wagner, R.R. and Thompson, T.E. (1979) Biochemistry 18, 538–543.
28 Franck, P.F.H., Op den Kamp, J.A.F., Roelofsen, B. and van Deenen, L.L.M. (1986) Biochim. Biophys. Acta 857, 127–130.
29 Raval, P.J. and Allan, D. (1984) Biochim. Biophys. Acta 772, 192–196.
30 Raval, P.J. and Allan, D. (1984) Biochem. J. 223, 555–557.