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Plasma membrane vesicles from BHK and HL60 cells treated with merocyanine 540 and iodoacetamide

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Treatment of BHK or HL60 cell lines with merocyanine 540 in the presence of the sulphydryl blocker iodoacetamide caused budding of the cell surface to release vesicles about 50–100 nm in diameter which accounted for up to 25% of the total surface membrane lipid. Smaller amounts of vesicular material were released in the presence of fluoride and merocyanine 540. The vesicles had a membrane lipid composition which was characteristic of other purified plasma membranes, with large amounts of sphingomyelin, phosphatidylserine and cholesterol and low proportions of phosphatidylinositol, phosphatidylcholine, triacylglycerol and cholesterol ester. This procedure for the isolation of vesicles should be a general method for the purification of plasma membrane components from a wide range of different cell types.

Introduction

Shedding of small vesicles from the surface of cells appears to be a rather common event in a large number of different cell types cultured in vitro or in vivo [1] but generally these vesicles represent a very small proportion of the cell and have not been exploited in most cases for the isolation and characterisation of plasma membrane. The mechanism of natural vesicle shedding is unknown although in some cases it may be a consequence of the fusion of internal membranes with the surface which occurs during exocytic events. One such case is represented by the process by which the transferrin receptor in maturing reticulocytes is concentrated in multivesicular bodies enclosed in endosomes and subsequently externalised by exocytosis [2]. Another example where addition of internal membrane to the cell surface provokes budding is in the case where the nuclear membrane of chicken erythrocytes fuses with the plasma membrane at elevated intracellular concentrations of Ca^{2+} [3].

Non-lytic shedding of vesicles from the surface of a variety of cells is promoted by treatment with detergents [4,5] phospholipids [6,7] and aldehydes or sulphydryl blockers [8], but except for platelets [7] and red cells the vesicles have not been well-characterised in terms of lipid and polypeptide composition. It is not even clear in some cases whether the vesicles are derived as buds of the cell surface encapsulating cytoplasm or whether they represent an exofacial fraction of the surface membrane without cytoplasm.

The best-characterised examples of vesicle shedding have been observed with red cells. Red cell vesicle release occurs in the presence of Ca^{2+} and ionophore A23187 [9] or under conditions of ATP depletion [10], in aged cells [11] and by treatment with detergents [5,12] or other amphiphiles [5]. In these cases, membrane shedding has been linked to conditions which alter the relative areas of inner and outer lipid leaflets, thus causing the increased membrane curvature which is an essential prelude to vesiculation [6,13,14]. However, other situations where the integrity of the membrane skeleton is affected rather than the lipid bilayer, also promote vesiculation of the red cell membrane, e.g., spectrin oxidation [15] or in hereditary disease of erythrocytes.

Merocyanine 540 (MC540) is a charged hydrophobic fluorescent compound which binds selectively to the outer leaflet of red cells and can cause a relative expansion of the area of that leaflet by about 2–3% [14]. This is associated with release of vesicles which contain in their membranes a representative sample of the lipids and proteins of the red cell membrane which are free to diffuse in the plane of the bilayer but not those (like spectrin and actin) which are part of the macromolecular complex comprising the membrane skeleton. We have shown recently that MC540 is also able to release vesicles from the surface of BHK cells

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and these vesicles possess the characteristic plasma membrane phospholipid pattern of high sphingomyelin and phosphatidylserine together with low phosphatidylinositol content [16]. However, polyacrylamide gel analysis of vesicles derived from ^{125}I-labelled cells showed no characteristic plasma membrane polypeptides which could be identified, suggesting either that these were not labelled by external ^{125}I or that there were no mobile proteins which could diffuse from the plasma membrane into vesicles.

A problem with MC540-induced vesicle release is that the yield is low, corresponding to only about 3–4% of total plasma membrane in BHK cells [16]. We reasoned that one of the factors which might act against exovesicle release was that BHK cells (like most other cell types) undergo a constant endovesiculation of their surface membrane and that if this endovesiculation could be inhibited then vesicle release might be increased. We therefore treated BHK cells with agents which reduce ATP levels since this represents one of the few effective methods of blocking endocytosis [17,18].

The results show that the yield of plasma membrane vesicles is dramatically increased under conditions where MC540 is added in the presence of iodoacetamide (a sulphydryl blocker which causes profound energy depletion) or in the presence of fluoride, which causes energy depletion without blocking sulphydryl groups.

Materials and Methods

Unless specified, all chemicals were obtained from Sigma, Poole, Dorset, UK.

Cell culture

BHK 21 cells were cultured on 3.5-cm dishes using the Glasgow modification of Minimal Essential Medium (GMEM), supplemented with 5% foetal calf serum (FCS) and tryptose phosphate broth as described previously [19]. HL60 cells were cultured in flasks using RPMI supplemented with 10% FCS. Initial cell concentrations were such that BHK cells were just confluent after 48 h and HL60 cells had reached a concentration of $2 \cdot 10^6$ cells/ml after 96 h). Cells were labelled to equilibrium in the presence of 5 μCi/ml of [^{3}H]acetate (NEN-Dupont) or 30 μCi/ml [^{32}P]phosphate (Amersham International).

Cell incubation and measurement of vesicle release

To measure the dependence of vesicle release on MC540 concentration, labelled cells were washed twice with phosphate-buffered saline (PBS) and then incubated at 37°C in PBS with 10 mM iodoacetamide. After 30 min MC540 was added to a range of final concentrations between 2–50 μM and the incubation was continued. After 60 min the medium was removed and centrifuged at $500 \times g$ for 5 min to remove detached cells and then at $100000 \times g$ for 2 h at 4°C to sediment the vesicles. The low- and high-speed pellets were resuspended in 0.5 ml of PBS and the lipids extracted with 1.9 ml of methanol/chloroform (2:1, v/v). The lipid from the cells remaining in the dishes, and from untreated cells was extracted directly with the same volume of solvent. Lipids were isolated from these extracts as described previously [19]. The extent of vesicle release was measured as the amount of chloroform-soluble radioactivity in the high-speed pellet compared with the total chloroform-soluble radioactivity in the original cells. In other experiments, cells were treated as above except that MC540 was added to a final concentration of 20 μM in all cases, and the release of vesicular material was measured at various time points up to 1 h or at 1 h in the presence of various concentrations of iodoacetamide.

Experiments were also carried out to compare the effects of iodoacetamide with fluoride, which caused ATP depletion without affecting sulphydryl groups. In these experiments, MC540 and the metabolic inhibitors were present throughout a 1 h incubation and vesicle release was measured by direct extraction of the low-speed supernate with chloroform/methanol without sedimentation of the vesicles.

Analysis of lipids

Radioactive lipids dissolved in chloroform were analysed as described previously [20]. In some experiments cholesterol mass [21] and the proportion of total lipid phosphorus [22] in each phospholipid class was measured for unlabelled BHK and HL60 cells.

Determination of cellular ATP

Following treatment with iodoacetamide or fluoride and MC540 as above, cells were gently washed with 5 ml of ice-cold PBS, after which 1 ml of the same buffer containing 150 μl of 12.5% perchloric acid was added and the cells were kept on ice for 10 min. The supernatant was removed into chilled 1.5 ml microfuge tubes and centrifuged at $10000 \times g$ for 1 min to remove any cell debris. After removal into a fresh tube, the supernatant was neutralized with a predetermined volume of a solution of triethanolamine/KOH (0.5 M and 2 M, respectively), such that the final pH of the sample was pH 7–8. Following centrifugation as above the supernatant was immediately frozen at −20°C. ATP levels in each sample were determined by the luciferase/luciferin assay [23].

Electron microscopy

Vesicles were released from BHK cells as described above and were sedimented at $100000 \times g$ for 1 h in the presence of 5 mg/ml defatted albumin in order to

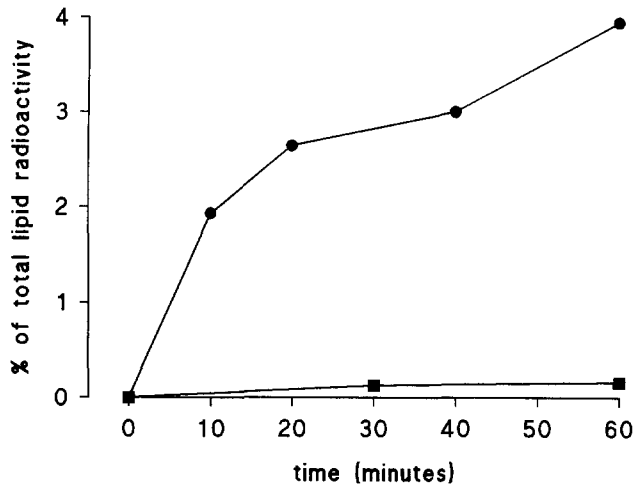

Fig. 1. Time-course of release of vesicles from BHK cells treated with iodoacetamide and MC540. [^{3}H]Acetate-labelled cells were preincubated for 30 min with 10 mM iodoacetamide and then treated for up to 1 h in the presence (circles) or absence (squares) of 20 μM MC540 at 37°C. The medium was removed at various time points and vesicles were isolated and analysed as described under Materials and Methods. The results shown were typical of those obtained in three other experiments carried out under the same conditions.

remove MC540. The pellet was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and then resuspended in 1% agarose. After treatment with 1% osmium tetroxide the sample was dehydrated and embedded in Araldite. Sections were post-stained with uranyl acetate/lead citrate before examination in a JEOL 1200EX electron microscope.

Results

After 30 min preincubation with iodoacetamide, ATP levels in [^{3}H]acetate-labelled BHK cells fell to about 5% of normal, the cells became rounded and some detached from the dish although only about 2% stained with Trypan blue. Addition of 20 μM MC540 caused half-maximal release of chloroform-soluble radioactivity from the cells in about 10 min (Fig. 1). Maximal release was obtained after about 1 h when as much as 6% of total cell lipid radioactivity was released (defined as material which was soluble in chloroform). 90% of the released material was sedimentable at $100000 \times g$ for 1 h but required 2 h centrifugation to achieve complete sedimentation. Electron micrographs of the pellet revealed that the material consisted mainly of small membrane vesicles 50–100 nm in diameter generally with dark-staining material within (Fig. 2). Figs. 3 and 4 show that maximal release was obtained with about 20 μM MC540 and 20 mM iodoacetamide. It was notable that iodoacetamide by itself gave very little release (Table I). *N*-Ethylmaleimide, another blocker of sulphydryl groups which causes ATP-depletion, produced similar effects to iodoacetamide in the presence of MC540 (results not shown). However, 30 mM fluoride which causes profound ATP-depletion without blocking sulphydryl groups caused about one third of the vesicle release seen with iodoacetamide in the presence of MC540. Similar amounts of vesicular material were obtained using HL60 cells exposed to the same conditions (results not shown).

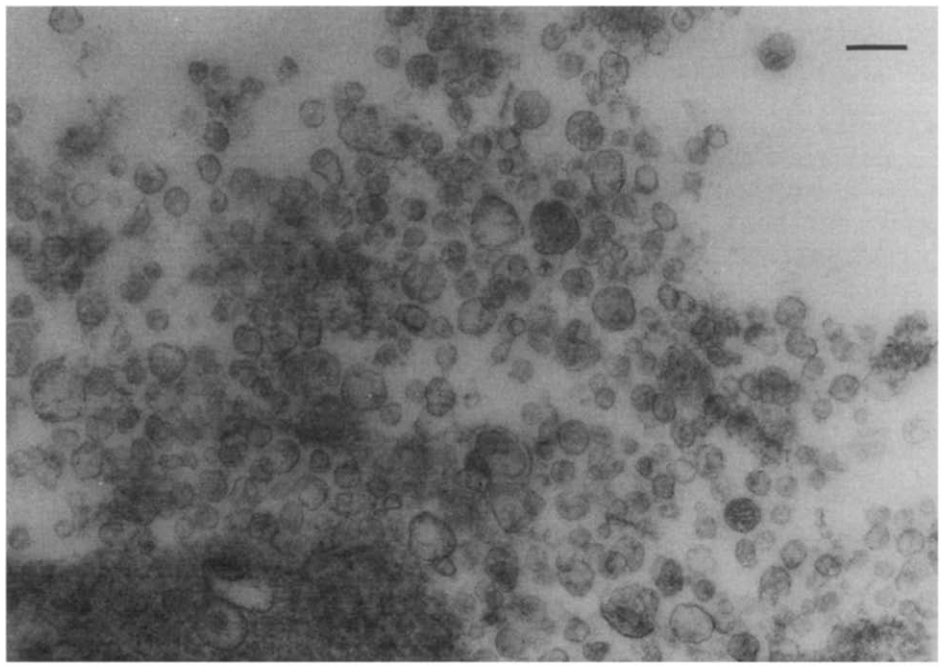

Fig. 2. Electron micrograph of vesicles released from BHK cells treated with iodoacetamide and MC540. Vesicles were released from BHK cells after incubation for 1 h as in the legend to Fig. 1. and were sedimented at $100000 \times g$ for 2 h. The pellet was examined in the electron microscope as described under Materials and Methods. Bar = 200 nm.

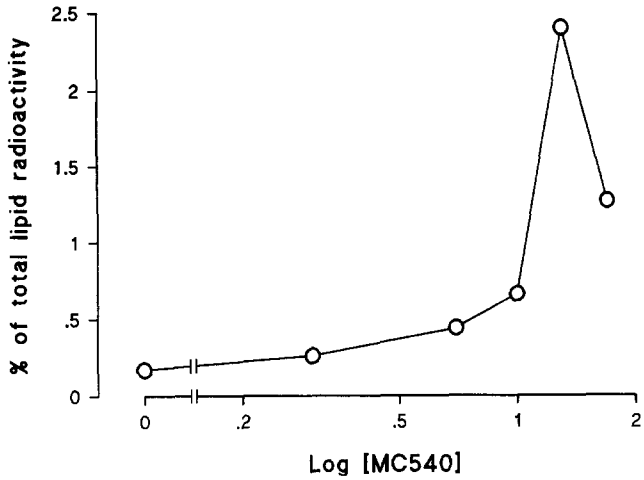

Fig. 3. The dependence of vesicle release on the concentration of MC540. Vesicles were isolated from BHK cells incubated as in the legend to Fig. 1 for 1 h in the presence of various concentrations of MC540 (in μM). Lipids were extracted and total radioactivity determined as above. The results shown are derived from a single experiment which was repeated on two further occasions with similar results.

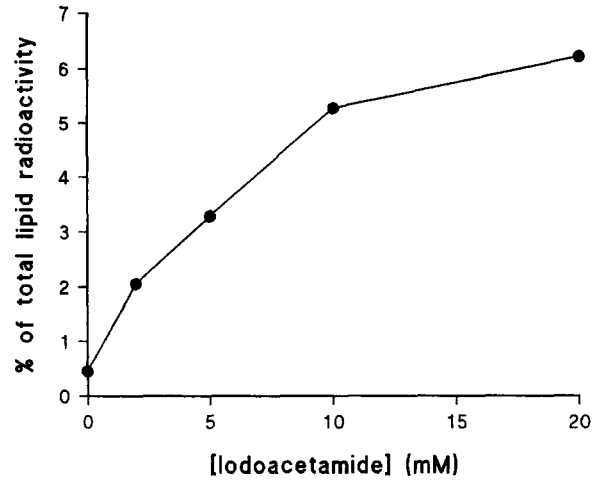

Fig. 4. The dependence of vesicle release on the concentration of iodoacetamide. Vesicles were isolated from BHK cells incubated as in the legend to Fig. 1 but using different concentrations of iodoacetamide in the presence of 20 μM MC540 for 1 h. Lipids were extracted and total radioactivity determined as above. The results shown are derived from a single experiment which was repeated on two further occasions with similar results.

The lipid composition of vesicles was compared with that of their progenitor cells by analysing the distribution of [^{3}H]acetate radioactivity (Fig. 5). This gave results for whole cells which were very similar to those obtained from mass determinations on phospholipids and cholesterol and it was accordingly assumed that the results for the vesicles were representative of lipid mass distribution. The overall lipid composition for BHK and HL60 cells was broadly similar (Fig. 5). For both cell types the vesicles contained about three times as much cholesterol, sphingomyelin and phosphatidylserine as the whole cells and showed corresponding reductions in phosphatidylcholine, phosphatidylinositol and triacylglycerol. Cholesterol ester was also greatly reduced in vesicles where it represented only 0.16% of total radioactivity compared with 0.6% in whole cells (results not shown in Fig. 5). Cardiolipin is a lipid which is characteristic of mitochondria and as expected was greatly reduced in vesicles compared with whole BHK cells. This reduction was not apparent in vesicles derived from HL60 cells but it appeared in this case that the cardiolipin spot was contaminated with another lipid which had a similar mobility to lactosylce-

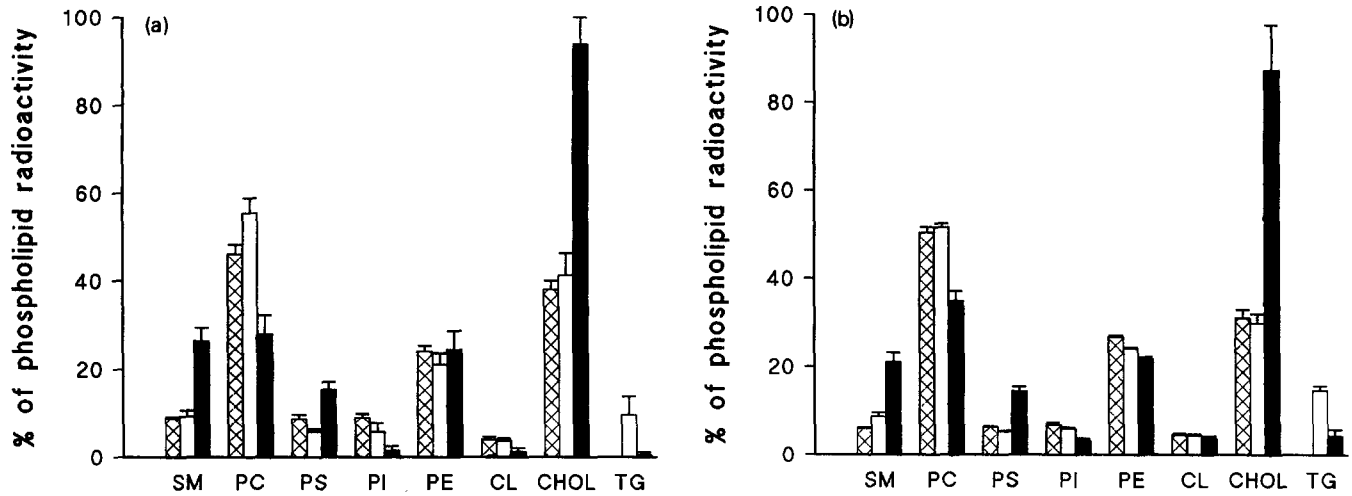

Fig. 5. The lipid composition of (a) BHK and (b) HL60 cells and vesicles derived from them. Vesicles were isolated after treatment of cells as in the legend to Fig. 1 with 20 μM MC540 for 1 h. Lipids from untreated cells (open bars) and from vesicles (solid bars) were separated and their radioactivity determined as described under Materials and Methods. Results are expressed as the proportion of total phospholipid radioactivity (means $\pm$ S.D. from four experiments) represented by each lipid class. Mass determinations of phospholipids and cholesterol from BHK and HL60 cells (hatched bars) gave results similar to the distribution of radioactivity.

TABLE I

Effects of energy depletion and sulphydryl blockers on release of vesicles from BHK and HL60 cells treated with MC540

Cells labelled to equilibrium with [^{3}H]acetate were incubated for 60 min at 37°C in phosphate-buffered saline (PBS) with and without 10 mM iodoacetamide or 30 mM NaF in the presence or absence of MC540. Vesicle release was measured in terms of the percentage of of chloroform-soluble radioactivity in the supernate after sedimentation of the cells. Chloroform-soluble radioactivity and ATP levels were measured in extracts of the cells (see Materials and Methods). Values represent the means ± S.D. from triplicate determinations in a single experiment which was repeated twice with substantially the same results. n.d. = not determined.

	ATP (% original)	Vesicle release (% total lipid)
PBS alone 0 min	100	0.10 ± 0.02
PBS alone 60 min	80 ± 4	0.24 ± 0.01
+ 10 mM iodoacetamide	1.5 ± 1.3	0.36 ± 0.01
+ 30 mM NaF	0.8 ± 1.0	0.48 ± 0.11
+ 20 μM MC540	74 ± 10	0.46 ± 0.02
+ 20 μM MC540 + iodoacetamide	1.2 ± 1.0	2.90 ± 0.37
+ 20 μM MC540 + fluoride	n.d.	1.17 ± 0.02

ramide (results not shown), a lipid previously shown to be a major component of human leukocytes [24].

Discussion

The results presented here extend our previous observations that MC540 can cause the release of surface vesicles from cells [14,16]. However, it is now clear that a much larger yield of vesicles can be obtained by pretreating the cells with iodoacetamide, which besides blocking sulphydryl groups, also causes a profound energy depletion. 30 mM fluoride, which is as effective as 10 mM iodoacetamide in reducing ATP levels, also increased vesicle release, but not as much as iodoacetamide (Table I). Thus it appears that although energy depletion per se does increase vesicle release in the presence of MC540, blocking of sulphydryl groups can be an important additional factor in increasing yields of vesicles. There have been previous reports that blockage of sulphydryl groups can destabilise the membrane skeleton in red cells [25] so it is possible that a similar effect on skeletal proteins in BHK and HL60 cells in conjunction with the ability of MC540 to selectively expand outer lipid leaflet area could explain the effects of iodoacetamide and MC540 on vesicle release.

The finding that iodoacetamide is so effective in increasing vesicle release recalls an earlier report by Scott that another suphydryl blocker, *N*-ethylmaleimide by itself induces vesicle release from a variety of cells [8]. However, we find that sulphydryl blockers by themselves have little effect and work well only in the presence of MC540 (Table I). Furthermore, the vesicles obtained by Scott were much larger and more variable in size than those released by MC540 in the presence of iodoacetamide. The small size of the MC540 vesicles is reflected by the large centrifugal force which is required to sediment them. These vesicles appear to be similar in size and morphology to the 'nanovesicles' previously shown to be released in addition to the larger 'microvesicles' from red cells treated with Ca^{2+} and ionophore A23187 [26]. The maximum amount of vesicle lipid which can be released from BHK or HL60 cells is equivalent to about 6% of total lipid (4% of total phospholipid) or about an order of magnitude larger than the amount released by MC540 in the absence of iodoacetamide [16].

MC540 was most effective at about 20 μM which is similar to the result observed with red cells, perhaps suggesting that vesiculation is related to a saturable binding of MC540 to the surface membrane [14]. However, MC540 is poorly soluble above 20 μM so it is not clear if binding is really saturable. The reduction in measured release of vesicles above 20 μM MC540 was a reproducible observation but the mechanism of this effect is unknown.

The lipid composition and time-course of release of vesicles from BHK cells pretreated with iodoacetamide was rather similar to that observed previously without iodoacetamide [16] although because of the much larger amounts of material produced in the presence of iodoacetamide, the values were more statistically significant. Again the composition was closely similar to that of the membrane envelope of Semliki Forest virus budding from the same cells [27] but contained about 25% more sphingomyelin and phosphatidylserine than a plasma membrane preparation derived by subcellular fractionation of BHK cells [28]. The lipid composition of vesicles from BHK and HL60 cells was very similar, and not very different from the composition of human red blood cells or platelet plasma membrane vesicles, with large amounts of sphingomyelin, phosphatidylserine and cholesterol measured but little phosphatidylinositol, cholesterol ester or triacylglycerol. This similarity in composition suggests that the lipid composition of plasma membranes from a wide variety of cells is relatively invariant. We are tempted to suggest that in many cases where reported plasma membrane lipid composition appears to differ significantly from the paradigm represented by the results obtained here, this could be due to contamination of plasma membrane preparations with intracellular membranes.

Although we cannot exclude the possibility that the material which enters the microvesicles represents the selection of certain mobile lipid components from a membrane which exhibits lateral inhomogeneity, there is little evidence for this in erythrocytes where the vesicles have the same lipid composition as cells [5,9–12] except with respect to the polyphosphoinositides [29], which probably interact specifically with proteins.

It is notable that sphingomyelin, phosphatidylserine and cholesterol all show a rather similar 2.5–3 fold enrichment in the vesicles compared with the whole BHK or HL60 cells confirming that these lipids are particularly characteristic of surface membranes. Indeed it is possible that a large proportion of these lipids reside only in plasma membrane or plasma membrane-derived organelles such as endosomes, which together could therefore account for 33–40% of total membrane lipid in BHK cells. In support of these calculations, a recent detailed analysis of plasma membrane lipid mass in CHO cells suggested that almost half of total cellular phospholipid was in plasma membrane and as much as two thirds of total cellular sphingomyelin and cholesterol was in plasma membrane alone [30]. This latter value is consistent with the observation that two thirds of sphingomyelin in BHK cells is available to an extracellular sphingomyelinase [31]. Thus for BHK cells the plasma membrane accounts for 22–26% of total cellular lipid and the maximum release of vesicles obtained in the presence of iodoacetamide and MC540 (6% of total cellular lipid) corresponds to about 25% of total plasma membrane lipid.

Phosphatidylinositol represented only about 2% of the vesicle phospholipids (based on labelling to equilibrium with either [^{3}H]acetate or [^{32}P]phosphate) and this is similar to the values obtained from red cells and from enveloped virus [27], suggesting that plasma membranes generally have low amounts of this lipid. This is probably a consequence of the conversion of phosphatidylinositol to polyphosphoinositides by the specific kinases which are known to be concentrated in plasma membranes [32,33]. Even the low levels of phosphatidylinositol obtained with BHK and HL60 vesicles are likely to be higher than normal values in the plasma membrane due to the likely dephosphorylation of polyphosphoinositides under the conditions of energy depletion employed here.

As noted in the Introduction, various methods have been used in the past to isolate vesicular fractions of cell membranes without causing cell lysis. Incubation with MC540 in the presence of suphydryl reagents seems to offer some advantages over these other procedures; thus it gives faster vesicle release than with phospholipid treatment, does not cause the multiple biochemical changes in the cells which are produced by Ca^{2+} and does not introduce potentially lytic concentrations of detergents. Iodoacetamide (but not fluoride) is however, likely to cause covalent modification of sulphydryl groups and consequent inactivation of some proteins. The major advantage of this procedure is that it enables the effective purification of plasma membrane lipids in relatively large yield without involving cell lysis and thus provides a datum line with which to judge the purity of other kinds of plasma membrane preparation from a wide variety of cells. It also produces a plasma membrane derivative which may (like enveloped virus) still maintain the molecular asymmetry of the original plasma membrane so that the asymmetric distribution of proteins and lipids could potentially be analysed more easily than in the intact cell.

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