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Echinocytosis and microvesiculation of human erythrocytes induced by insertion of merocyanine 540 into the outer membrane leaflet

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Echinocytosis and release of microvesicles from human erythrocytes treated with the impermeant fluorescent dye merocyanine 540 (MC540) has been correlated with the extent of dye binding to intact cells and ghosts. At 20°C binding appeared to saturate at about $9.3 \cdot 10^6$ molecules per cell (3.6 mol/100 mol phospholipid), equivalent to an expansion of the outer leaflet lipid area of about 2.7%. Stage 3 echinocytes were formed upon binding of $(3–4) \cdot 10^6$ molecules of MC540/cell (about 1.3 mol/100 mol phospholipid), equivalent to an expansion of the outer leaflet lipid area of about 1.0%. Negligible release of microvesicles was observed with MC540 at 20°C. Binding of MC540 to permeable ghosts was approximately twice that to cells suggesting that there was no selective binding to the unsaturated (more fluid) phospholipids which are concentrated in the inner lipid leaflet of the membrane. At 37°C apparent maximal binding of MC540 was about 3.2 mol/100 mol phospholipid and correlated with the maximal release of microvesicles from the cells as measured by release of phospholipid and acetylcholinesterase. These results are discussed in relation to the bilayer couple hypothesis of Sheetz and Singer (Proc. Natl. Acad. Sci. USA 71 (1974) 4457–4461).

Introduction

The bilayer couple hypothesis of Sheetz and Singer [1], which built on earlier work of Deuticke [2] and Evans [3], sought to explain drug-induced changes in erythrocyte morphology in terms of the preferential intercalation of drug molecules into one of the leaflets of the membrane lipid bilayer, leading to differential expansion of that leaflet and a consequent local curvature of the membrane. Support for this attractive and simple idea has grown over the last decade [4–7] and the hypothesis has been generalised to include cases where differential contraction of either of the lipid leaflets by removal of lipid also gives rise to predictable changes in morphology [8–11]. However, estimates of the amount of outer lipid leaflet expansion necessary to induce echinocytosis have ranged from 1.5% to 4% [4–7], generally higher than theoretical estimates of 0.7% [7] or 0.4% [12] based on geometrical considerations. Under some circumstances echinocytosis induced by outer lipid leaflet expansion [13,14] or inner leaflet contraction [15,16] can proceed to the point where membrane material is lost to the medium in the form of microvesicles, but there appears to be no information regarding the precise relative change of membrane leaflet area necessary to cause microvesiculation.

We were initially interested in the binding of MC540 to erythrocytes because of reports [17–19] that this hydrophobic fluorophore only bound to cells which had undergone a rearrangement of their normal asymmetric distribution of phospholipids resulting in an increased fluidity of the outer lipid leaflet. It was suggested that phosphatidylethanolamine and phosphatidylserine which are rich in unsaturated (fluid) fatty acids and which are normally mainly confined to the inner lipid leaflet of the erythrocyte membrane [20] can under some circumstances migrate to the outer leaflet and there promote the binding of MC540 which was presumed to interact strongly with fluid lipids but not with gel-phase lipids [18]. The results of our experiments do not support this suggestion and indicate that MC540 binds equally well to inner and outer leaflet lipids. However, MC540 proved to be an appropriate tool with which to relate the degree of outer leaflet expansion to

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echinocytosis and microvesicle release, for the following reasons. Firstly, insertion and orientation of MC540 in the membrane can be accurately analysed. Secondly, MC540 selectively inserts into the outer membrane leaflet because its transbilayer migration is very slow [18]. Thirdly, MC540 is a compact and rigid molecule so that from its molecular volume its membrane expanding effect can be easily calculated.

Methods

MC540 was obtained from Sigma Chemical Co. and was dissolved at a concentration of 1 mM in ethanol or 10 mM in dimethylsulphoxide. Egg phosphatidylcholine and dimyristoylphosphatidylcholine were also obtained from Sigma and were sonicated at a concentration of 1 mM in 150 mM NaCl, 10 mM Hepes-NaOH buffer, 0.1 mM EDTA (pH 7.4) using an MSE sonicator (12 μ peak to peak amplitude).

Emission spectra were measured in a Perkin-Elmer luminescence spectrometer using an excitation wavelength of 540 nm. The spectrum of 1 ml of a 100 nM solution of MC540 was analysed in 150 mM NaCl, 10 mM Hepes-NaOH buffer (pH 7.5) in the presence of (a) washed erythrocyte membranes (100 nmol lipid phosphorus); (b) 100 nmol egg phosphatidylcholine sonicated for 5 s; (c) 100 nmol of dipalmitoylphosphatidylcholine sonicated as for (b) but for 20 s; (d) 0.5 mg of bovine serum albumin. The emission spectrum of 100 nM MC540 was also measured in various organic solvents including a range of homologous *n*-alkanols, chloroform and benzene.

Fresh human blood (20 ml) was collected in 150 mM NaCl containing 1 mM EGTA and erythrocytes were washed three times by centrifugation in 50 ml of 150 mM NaCl, 10 mM Hepes-NaOH buffer (pH 7.4). White ghosts were prepared from 1 ml of packed cells by lysis in 50 ml of ice-cold 10 mM Tris-HCl buffer (pH 7.6) containing 0.1 mM EGTA followed by three washes by centrifugation ($30\,000 \times g$ 10 min) in the same solution.

Various concentrations of MC540 (0–20 μM) were added to washed human erythrocytes (20 μl of packed cells) or packed ghosts (20 μl) suspended in 1-ml aliquots of 150 mM NaCl 10 mM Hepes-NaOH buffer (pH 7.5) in 1.5 ml Eppendorf plastic tubes. Parallel control samples containing MC540 but no cells or ghosts were also prepared to correct for non-specific binding to the tubes. After incubation at 20 °C for 5 min the samples were centrifuged in an Eppendorf bench centrifuge at 14000 rpm for 5 min and the supernatant solution was removed. The pellet was resuspended in 1 ml of the original buffer and a 50-μl sample of the suspension was added to 1 ml of *n*-butanol and vortexed for 10 s. The amount of MC540 in the butanol solution was measured from its fluorescence emission at 580 nm in comparison with a standard 100 nM solution of MC540 in butanol using excitation at 540 nm in a Perkin-Elmer LS-5 fluorimeter. Reextraction of the residual cell pellet with butanol, ethanol or 2:1 methanol/chloroform showed that the initial butanol extract of the cells accounted for > 99% of MC540 originally bound and that the results were not significantly (< 2%) affected by contamination with unbound dye. The remaining resuspended cells and ghosts were extracted with 2:1 methanol/chloroform and their total phospholipid content was assayed as described previously [21].

In some experiments separate samples of cells were incubated with MC540 as above and echinocyte formation at each concentration of dye was assessed by light microscopy of the suspension between two plastic cover slips. The degree of echinocytosis was quantified according to the nomenclature of Bessis [22].

Experiments to measure release of microvesicles were carried out as above but at 37 °C in glass tubes and using a total volume of 4 ml. Microvesicle release was quantified in terms of phospholipid and acetylcholinesterase lost from the cells as described previously [13,21]. Cell lysis was measured spectrophotometrically (418 nm) and was expressed as the percentage of total cell haemoglobin which was not sedimentable after high speed centrifugation.

Estimates of the dimensions of the MC540 molecule were made from a computer display using Desktop Molecular Modeller (Oxford Electronic Publishing, Oxford University Press, Oxford, U.K.). The dimensions of the hydrophobic part of the molecule were found to be $18.5 \times 9 \times 4.5$ Å, giving a cross-sectional area of about 40 Å^2. These values agree with a calculation of the membrane volume of the hydrophobic portion of MC540 based on the work of Kita and Miller [23], which gave a value of 700 Å^3.

Results

Binding of MC540 to cells and membranes at 20 °C (Fig. 1) increased with the concentration of dye but appeared to saturate at about 15 μM. It was not practicable to investigate the effects of concentrations higher than 20 μM because the dye became increasingly insoluble. Even at lower concentrations some problems were experienced with non-specific binding of dye to the tubes but this was largely overcome by the use of suitable controls. The maximum amount of dye bound to cells was 3.6 ± 0.4 mol/100 mol lipid phosphorus and the equivalent value for the ghosts (which were freely permeable to small molecules [24]) was 7.4 ± 0.9. With increasing concentration MC540 induced a progressive change in shape of the cells from discocyte to spheroechinocyte as shown in Fig. 2. Stage 3 echinocytes were produced when binding of MC540 was (3–4) $\cdot 10^6$ molecules per cell.

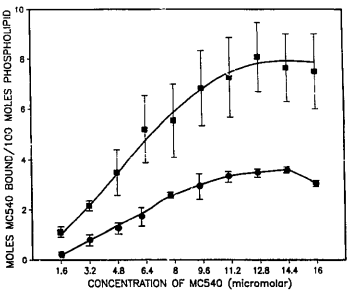

Fig. 1. Binding of MC540 to human erythrocytes and ghosts at 20 °C. Various concentrations of MC540 were added to washed human erythrocytes or erythrocyte ghosts and dye binding was measured as described under Methods. Results are shown as means ± S.D. from four experiments with different samples of cells (●) and ghosts (■).

Cell morphology was assessed at 20 °C because it was easier to maintain a constant temperature close to ambient on the microscope slide and the same temperature was used when measuring binding of MC540 to cells and ghosts so that a direct comparison under the same conditions could be made of MC540 binding and shape changes. Microvesicle release was not seen below 20 °C but was maximal at 37 °C so that the latter temperature was used in those experiments where microvesicles were measured. MC540 was more soluble at the higher temperature so that it was possible to employ higher concentrations of the dye than at 20 °C.

Release of microvesicles from cells incubated at 37 °C for 10 min, as judged from phospholipid and acetylcholinesterase activity in the supernatant solution, was maximal at 20 μM MC540 using a 10-min period of incubation (Figs. 3b and 3c). No microvesicles were released below 5 μM MC540 and the apparent maximum release of microvesicles coincided with maximal binding of the dye (about 3.2 mol/100 mol phospholipid) (Fig. 3a). Approx. 10% of total cell phospholipid and 20% of acetylcholinesterase were released under these conditions where cell lysis was 2–3% (Fig. 3d) and 90% of the released material could be sedimented after centrifugation for 1 h at 100000 × *g*. Like microvesicles released from erythrocytes by other procedures [13,21,25], these microvesicles contained Band 3 but were largely free of actin and spectrin as judged by electrophoresis on polyacrylamide gels (Fig. 4) although not significantly different from cells in their phospholipid composition (Table I).

In order to obtain an indication of the site at which MC540 bound, the spectrum of the dye was analysed when bound to ghosts and compared with the spectrum of the dye when bound to bovine serum albumin, egg phosphatidylcholine, or dipalmitoylphosphatidylcholine (Fig. 5) and when dissolved in solvents of different polarity (Fig. 6). As shown previously for the absorption spectrum [26], the fluorescence emission maximum of MC540 was red-shifted in solvents of decreasing polarity (Fig. 5) and it was thus possible to assess the effective dielectric constant of the environment of MC540 in a variety of situations. When interacting with erythrocyte membranes the emission maximum of MC540 was shifted from 565 nm in the aqueous medium to 583.3 nm which indicates an environment for the dye which was similar in hydrophobicity to *n*-decanol. An identical change is observed when MC540 interacts with phospholipid vesicles whereas binding to albumin (a protein which has strong binding sites for hydrophobic

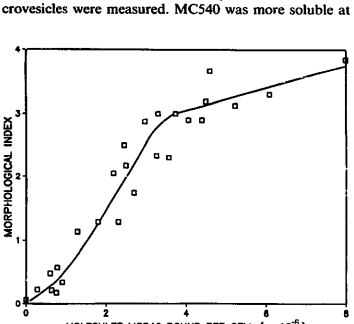

Fig. 2. Morphological index of erythrocytes treated with MC540 at 20 °C. Cells were exposed to MC540 in an experiment similar to that shown Fig. 1. Results from four experiments using haematocrits between 0.5% and 2% are combined to illustrate the relationship between MC540 bound per cell and the stage of echinocytosis according to the nomenclature of Bessis [22].

TABLE I

Phospholipid composition of (a) untreated red cell ghosts, (b) ghosts from MC540-treated cells, (c) microvesicles

Phospholipids were extracted and analysed as described previously [21]. Results represent mole percent of total phospholipid and are shown as means ± S.D. from three experiments, each carried out with duplicate samples.

	Phospholipid composition		
	(a)	(b)	(c)
Phosphatidylethanolamine	29.9 ± 0.3	28.8 ± 0.9	28.8 ± 1.9
Phosphatidylserine	15.6 ± 1.9	14.0 ± 0.5	12.9 ± 2.0
Phosphatidylcholine	28.4 ± 0.7	29.2 ± 1.3	30.2 ± 3.3
Sphingomyelin	23.2 ± 1.4	25.0 ± 1.8	23.8 ± 3.4

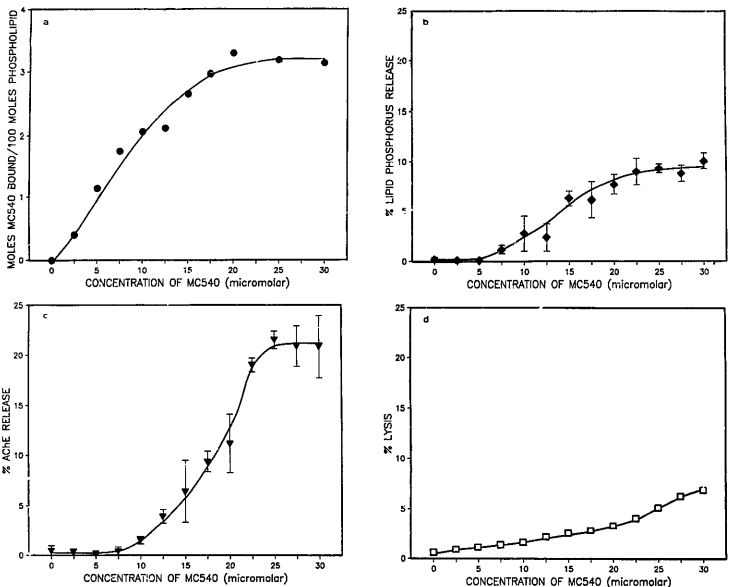

Fig. 3. Binding of dye and release of phospholipid and acetylcholinesterase from erythrocytes treated with MC540 at 37 °C. After incubation with various concentrations of MC540 for 10 min at 37 °C as described under Methods, cells were sedimented and binding of dye (a) was measured as described above. The supernatant solution was assayed for phospholipid (b), acetylcholinesterase activity (c) and haemoglobin release (d). Results shown are the means ± S.D. from four experiments with different samples of cells. Values for phospholipid and acetylcholinesterase release are corrected for lysis.

molecules) gives an emission maximum at 574 nm, indicative of a relatively hydrophilic environment for the MC540 (Fig. 5). These data indicate that the major site of MC540 interaction with membranes is the hydrophobic lipid region and not with membrane protein. The magnitude of the fluorescence maximum of MC540 when interacting with egg phosphatidylcholine was generally larger than with dipalmitoylphosphatidylcholine but this difference became progressively less when the dipalmitoylphosphatidylcholine was sonicated for longer periods. Similar findings were reported by Schlegel et al. [17] who suggested that this was due to enhanced binding of MC540 to very small vesicles whose acute curvature allowed the dye to enter the bilayer. However, it was clear in our hands that it required much more sonication to obtain a clear solution from dipalmitoylphosphatidylcholine than from egg phosphatidylcholine and this could have the consequence that the proportion of the phospholipid present as unilamellar vesicles was relatively less with dipalmitoylphosphatidylcholine. Since it is apparent that MC540 interacts only with phospholipid monolayers which are exposed to the external aqueous medium this factor rather than any intrinsic difference in ability to bind MC540 between saturated and unsaturated phospholipids could be responsible for the differences observed.

Discussion

Normal human erythrocytes bind significant quantities of MC540, equivalent to a maximum of 3.6 mol/100 mol phospholipid or to about $9 \cdot 10^6$ molecules per cell

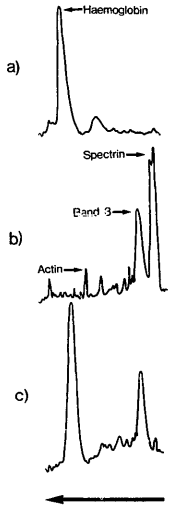

Fig. 4. The polypeptide pattern of vesicles from MC540-treated erythrocytes. The polypeptide patterns of (a) cytosolic proteins, (b) erythrocyte membrane proteins and (c) microvesicle proteins were analysed by SDS-PAGE (11% w/w gel) using the method of Laemmli [42] and a Bio-Rad Minigel apparatus. Bands were visualised with Coomassie-brilliant blue stain ard scanned using a Bio-Rad 1650 Scanning Densitometer linked to a Shimadzu C-R3A integrator.

(Fig. 1). This finding appears to conflict with the work of Schlegel and coworkers [17] who claimed that normal erythrocytes do not bind this dye, but their measurements were made in the presence of serum which contains albumin and lipoprotein that would certainly compete with cells for binding MC540. However, in the absence of serum the binding of MC540 to erythrocytes observed by Schlegel et al. was as much as $3 \cdot 10^7$ molecules per cell, more than 3-fold greater than our values. Part of this apparent discrepancy might be due to the use of avian erythrocytes by Schlegel et al. but more significantly, these authors used low ionic strength buffer which could have allowed extra ionic binding of MC540 to the cells.

Erythrocyte ghosts, which in contrast to the intact cells are permeable to MC540, bind twice as much MC540 as cells, for the same amount of lipid phosphorus (Fig. 1) and this suggests that inner leaflet phospholipid has no greater affinity for the dye than

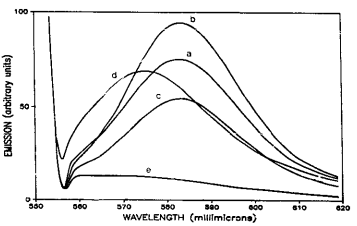

Fig. 5. The spectrum of MC540 in the presence of an excess of erythrocyte plasma membranes, egg phosphatidylcholine, dipalmitoylphosphatidylcholine or bovine serum albumin. The emission spectrum of 1 ml of a 100 nM solution of MC540 was analysed in 150 mM NaCl, 10 mM Hepes-NaOH buffer (pH 7.5) in the presence of (a) washed erythrocyte membranes (100 nmol lipid phosphorus), (b) 100 nmol egg phosphatidylcholine sonicated with a sonic probe for 5 s, (c) 100 nmol dipalmitoylphosphatidylcholine sonicated as for (b) but for 20 s, (d) 0.5 mg of bovine serum albumin, (e) buffer alone.

outer leaflet phospholipids despite the considerable difference in content of unsaturated fatty acids between the two leaflets. Only at the lowest concentration of MC540 (1.6 μM) was there a slight indication of a higher affinity of ghosts for the dye and this could suggest preferential binding to inner leaflet lipid. These findings support our earlier conclusions regarding the

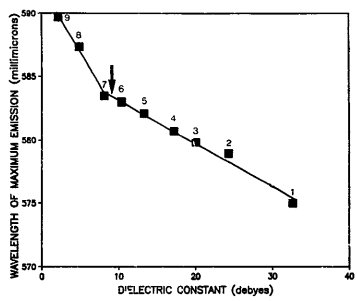

Fig. 6. The dependence of the fluorescence emission peak wavelength of MC540 on the solvent polarity. The spectrum of a 100 nM solution of MC540 was analysed in solvents of different polarity using a Perkin-Elmer LS-5 luminescence spectrometer. Fig. 6 illustrates the relationship between solvent dielectric constant and the wavelength of maximum fluorescence following illumination at 540 nm. 1, methanol; 2, ethanol; 3, *n*-propanol; 4, *n*-butanol; 5, *n*-hexanol; 6, *n*-octanol; 7, *n*-decanol; 8, chloroform; 9, benzene. The arrow marks the wavelength of maximum emission for MC540 in the presence of an excess of erythrocyte membrane.

interaction of MC540 with ghosts and microvesicles from human erythrocytes [27] but do not support the claims of Schlegel et al. [17–19] that inner leaflet but not outer leaflet lipid binds MC540. Our experiments comparing the spectral changes undergone by MC540 after addition of saturated or unsaturated lecithins (Fig. 5) also suggest that any differences in the interaction of the dye with lipids are not due to differences in saturation *per se* but rather to variations in the micellar form of the phospholipid. The observations that ghosts bind about twice as much MC540 as intact cells and that binding to the erythrocytes does not increase upon prolonged incubation are further confirmation that once bound to the surface of intact cells MC540 has little tendency to undergo transbilayer migration. It is noteworthy that binding of MC540 to erythrocytes or to ghosts is a saturable process (Figs. 1 and 3a), perhaps limited by the surface pressure of membrane lipids, by the increase in surface charge due to the introduction of anionic dye into the bilayer [28] or by specific constraints imposed by the underlying membrane skeleton [29]. Thus factors which affect surface charge, pressure or skeletal interactions may in turn affect the binding of dye and the observed changes in fluorescence.

There has been considerable speculation regarding the precise mode of interaction of MC540 with cell membranes, particularly among those workers interested in the small increase in fluorescence which parallels the action potential in stimulated nerves exposed to MC540 [30]. It has been suggested that this change in fluorescence reflects the rotation of the MC540 molecule from a position parallel to the fatty acid chains of membrane lipids to a position at right angles to those molecules [31,32]. It is not clear what are the relative proportions of these two orientations but it would require only a very small proportion of the total bound MC540 to change its orientation in order to explain the small changes observed.

The fluorescence spectrum and hence the environment of MC540 in erythrocyte membranes appears to be almost indistinguishable from that in a sonicate of pure phospholipid (Fig. 5) and this environment seems to be a hydrophobic one which corresponds to a solvent similar in polarity to *n*-decanol, with a dielectric constant of about 9 (Fig. 6). This value is very similar to that measured by Lelkes and Miller from absorption spectra [26] although curiously, these workers concluded that the dye was most likely to be oriented parallel to the surface of the membrane with the chromophore interacting mainly with phospholipid headgroups and apparently accessible to the aqueous medium. On the basis of the spectral evidence we consider it to be more likely that most of the bound dye is oriented so that a large part of the long chromophore is associated with the most hydrophobic region of the membrane, i.e., where the long axis of the molecule lies parallel with the fatty acid chains of membrane phospholipids. Although there are obvious difficulties in comparing the environment of a dye in an anisotropic membrane environment with the same dye in an isotropic solvent, it seems to us probable that if MC540 really had major interactions with phospholipid headgroups as suggested by Lelkes and Miller, then its spectrum in the membrane would not resemble its spectrum in decanol.

Assuming that intercalation of MC540 between the fatty acid chains of outer leaflet phospholipids accounts for most of the binding of MC540 to intact cells it is possible to calculate the expansion of the outer leaflet corresponding to maximum binding of the dye. Thus taking the cross-sectional area of the MC540 molecule as 40 $Å^2$ (based on measurements of molecular models) and assuming that one cell contains $4.3 \cdot 10^{-6}$ mol of phospholipid [33] and has a surface area of $133 \cdot 10^8$ $Å^2$ [11] then introduction of 3.6 mol MC540/100 mol total phospholipid into the outer lipid leaflet will expand its area by 2.7%. The amount of dye binding necessary to convert all the cells to stage 3 echinocytes is about a third of this value, i.e., a 1.0% expansion. This is less than the lowest estimates previously reported for outer lipid leaflet expansion in echinocytosis [4–7] but greater than estimates obtained theoretically [7,12]. The difference between the experimental values and the theoretical estimates may be due in part to an expansion of the inner lipid leaflet in response to outer leaflet expansion, particularly when the membrane-expanding agent has a long hydrophobic region (e.g., phosphatidylcholine) which can penetrate to the core of the bilayer [10]. Nevertheless, the clear relationship between MC540 binding and shape change is further evidence that the binding that we have measured is not superficial but involves the insertion of specific numbers of dye molecules between the fatty acid chains of the outer leaflet phospholipids as envisaged in the bilayer couple hypothesis [1]. We cannot be certain that all the MC540 is bound in the same region of the membrane; it is possible that some of the dye is bound at superficial sites of hydrophilic character which would not give rise to a visible fluorescence. However, in this case the degree of membrane expansion required to cause the observed morphological changes would be even less than the 1% value calculated above.

At 37°C and at levels of MC540 binding beyond those sufficient to cause echinocytosis the cells respond by shedding microvesicles into the medium (Fig. 3) in a process analogous to that induced by incubation with dimyristoylphosphatidylcholine which is presumed to also act by intercalating between membrane phospholipid fatty acid chains so as to cause unilateral lipid leaflet expansion. However, much less acetylcholinesterase was released by MC540 compared with that seen with dimyristoylphosphatidylcholine [13] (20% versus 70%) and there was no time-lag in the release

with MC540 (results not shown) unlike the latter compound. The faster effect of MC540 on microvesicle release could be due to a more rapid partition of the dye into membranes because the concentration of the dye monomer would be much higher than in the case of dimyristoylphosphatidylcholine, most of which would be in the form of liposomes. The extent of microvesicle release with MC540 and the acetylcholinesterase specific activity of the microvesicles seemed to be much more similar to the equivalent values of microvesicles from cells treated with A23187/Ca^{2+} [21] or with glycocholate [25] than from cells exposed to dimyristoylphosphatidylcholine [13]. Possibly some of the acetylcholinesterase is released in a soluble form in the latter case.

As expected, maximum release of microvesicles correlated with maximum binding of MC540 but cell lysis continued to increase at higher concentrations of dye. If as seems likely, a membrane fusion event is involved in the release of microvesicles then this process only takes place when the expansion of the outer leaflet of the membrane is greater than 1% and is maximal when the expansion is about 2–3%. The marked temperature dependence of microvesicle release, with little release seen below 20 °C may be a reflection of a temperature-sensitive decrease in lipid fluidity which affects membrane fusion.

The conclusion that as little as a 1% change in relative area of inner and outer leaflets of the erythrocyte membrane can cause echinocytosis and that a 1–3% change can precipitate vesiculation events has further consequences for erythrocytes and other cells. Thus the echinocytosis and microvesiculation which results from Ca^{2+} entry into human erythrocytes [15,21] or the echinocytosis caused by energy depletion might be explained by a relative diminution of inner lipid leaflet area due to activation of a membrane-bound phospholipase C [34] or phosphatase [35] specific for polyphosphoinositides which account for about 2–3% of inner leaflet lipids. Ferrell and Huestis [35] have estimated that the diminution of the inner monolayer area when phosphatidylinositol 4,5-bisphosphate and phosphatidate are dephosphorylated by energy depletion is about 0.65%. A larger effect would be expected if all the polyphosphoinositides were converted to diacylglycerol by an endogenous phospholipase C especially as diacylglycerol can potentially diffuse into the outer leaflet causing an additional increase in the difference between inner and outer leaflet areas [9]. Phospholipases C similar to that in the red cell are present in the plasma membranes of other cell types where they appear to be involved in the transduction of certain extracellular signals [36], although it is not known if inositide breakdown in these cells has any direct effect on plasma membrane morphology. Certainly there is some evidence in red cell ghosts that the levels of polyphosphoinositides can influence the shape and deformability of membranes [37]. On the other hand, Ca^{2+}-induced decrease in membrane stability has been explained in terms of a decreased association between membrane skeletal proteins mediated either by Ca^{2+}-calmodulin [38] or by Ca^{2+}-dependent activation of a phospholipase C which attacks polyphosphoinositides [39]. However, it is interesting to speculate that the unilateral insertion of lipids into membranes which appears to occur during lipid biosynthesis in the endoplasmic reticulum [40] and during the process of phospholipid redistribution mediated by cytoplasmic carrier proteins [41], results in predictable changes in membrane curvature which influence subsequent fusion events in those membranes.

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