Biochem. J. (1990) 271, 831–834 (Printed in Great Britain)

Restricted diffusion of integral membrane proteins and polyphosphoinositides leads to their depletion in microvesicles released from human erythrocytes

Carla HAGELBERG and David ALLAN*
Department of Physiology, University College and Middlesex School of Medicine, University College London, Rockefeller Building, University Street, London WC1E 6JJ, U.K.

The protein and phospholipid composition of microvesicles released from normal human erythrocytes after ATP depletion, on aging or by treatment with merocyanine 540, dimyristoyl phosphatidylcholine or Ca^{2+}/ionophore A23187 has been compared with the composition of the original cell membrane. It has been shown that these microvesicles are depleted of band 3, glycophorin and phosphatidylinositol 4,5-bisphosphate relative to phospholipid by 40% or more. These data are interpreted to mean that less than half of these membrane components are free to diffuse laterally in the lipid bilayer. Acetylcholinesterase was found to be enriched 2–3-fold in microvesicles, possibly because the removal of non-diffusing proteins from the vesiculating region of the lipid bilayer allows more space for freely diffusing proteins like acetylcholinesterase to enter the microvesicle membrane.

INTRODUCTION

Microvesicle release from echinocytic human erythrocytes occurs naturally during the process of aging [1] and in certain disease states [2,3]. It can also be induced *in vitro* under a variety of different conditions such as calcium loading [4], ATP depletion [5], spectrin oxidation [6] or through the intercalation of certain amphipaths into the outer lipid bilayer [7–9]. In all these cases the composition of the microvesicle membrane resembles that of the original cell, with the notable exception that those membrane proteins which form part of the non-diffusing skeletal complex (e.g. spectrin and actin) are not found in the microvesicles. It has been shown that, in echinocytic blebs, the lateral mobility of antigens is not restricted as it is in the rest of the membrane [10]. This suggests that only those components of the erythrocyte membrane which are free to diffuse in the plane of the bilayer (e.g. lipids and those membrane proteins which are associated with lipid and not with the skeleton) would be expected to be present in the microvesicles. Band 3 does appear among the proteins of the microvesicle membrane, so that at least a fraction of this protein must be diffusible. It is well known that band 3 protein can associate (via ankyrin and possibly band 4.1) with the skeletal complex [11,12], but there appear to be too many molecules of band 3 for them all to be bound to ankyrin and band 4.1 [13], so it might be expected that a fraction of band 3 would be free to diffuse in the lipid bilayer.

We thought that it should be possible to obtain a quantitative measure of the proportion of band 3 molecules which were freely diffusing (i.e. not bound to skeletal proteins) by determining the relative amount of band 3 which partitioned into microvesicles produced under mild conditions. Furthermore, such measurements might reveal differences in the extent of attachment of band 3 under various conditions of microvesicle release. Similar considerations would apply to other components of the bilayer portion of the membrane, such as glycophorin and polyphosphoinositides, which may also have interactions with the membrane skeleton [14].

MATERIALS AND METHODS

Merocyanine 540 (MC540), dimyristoylphosphatidylcholine (DMPC), iodoacetamide, deoxyglucose and Ca^{2+} ionophore A23187 were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Preparation of cells and ghosts

Fresh human blood was collected from healthy volunteers in 150 mM-NaCl containing 2 mM-EDTA (pH 7.4) and used within 1 day of collection. The erythrocytes were sedimented by centrifugation at 500 *g* for 5 min and were washed twice with 150 mM-NaCl (with the removal of the buffy coat) and once with the incubation buffer [150 mM-NaCl, 10 mM-Hepes/NaOH/0.1 mM-EDTA, pH 7.4 (Hepes-saline)].

Ghosts were prepared by lysing the cells with ice-cold 20 mM-Tris/HCl/2 mM-EDTA, pH 7.4. Membranes were sedimented and washed once with this buffer prior to phospholipid analysis or three times prior to polypeptide analysis.

Protein analysis

Polypeptides were analysed by SDS/PAGE using the Laemmli system [15]. Gels were stained with Coomassie Brilliant Blue or periodate/Schiff reagent [16]. Quantification of the bands was achieved by scanning the gels with a Bio-Rad 1650 scanning densitometer linked to a Shimadzu C-R3A integrator. Acetylcholinesterase (AChE) activity was determined according to the method of Ellman *et al.* [17].

Lipid analysis

Lipids were extracted from membranes with chloroform/methanol (1:2, v/v) [18] and were separated by t.l.c. on Merck silica gel 60 plates run in chloroform/methanol/acetic acid/water (75:45:12:2, by vol.) [19]. Polyphosphoinositides were extracted as above, but with the addition of HCl to the extract [20], and were separated on oxalate-impregnated plates [1% in methanol/water (2:3, v/v)] using chloroform/acetic acid/methanol/

Abbreviations used: MC540, merocyanine 540; DMPC, dimyristoyl phosphatidylcholine; AChE, acetylcholinesterase.

* To whom all correspondence should be addressed.

acetone/water (40:12:13:15:7, by vol.) as the solvent system [21]. Phospholipid spots were scraped from the plate and digested with 70% (v/v) $HClO_4$ at 180 °C for 1 h, and lipid phosphorus was assayed by the method of Bartlett [22].

Radioactive spots were visualized by autoradiography, excised and digested with 70% $HClO_4$. After dilution with 10 ml of water the samples were counted for radioactivity in a liquid scintillation analyser. The phosphorus assay for PtdIns(4)*P* was unreliable due to a variable contamination with an unknown lipid; therefore PtdIns(4)*P* was quantified from its radioactivity, assuming that the specific radioactivity is half that of PtdIns(4,5)P_2 [23].

Labelling of erythrocytes with ^{32}P

Cells at a haematocrit of 20% were incubated for 18 h at 37 °C in Hepes-saline containing $MgCl_2$ (1 mM), glucose (10 mM), gentamicin (100 μg/ml) and carrier-free [^{32}P]phosphate (5 μCi/ml) (Amersham International).

T.l.c. lipid analysis of ^{32}P-labelled microvesicles, ghosts and MC540 ghosts confirmed previous results, i.e. the only erythrocyte lipids to be labelled with ^{32}P are those bearing monoester phosphate groups, i.e. phosphatidic acid, PtdIns(4)*P* and PtdIns(4,5)P_2 [24].

In some experiments the specific radioactivities of ATP and AMP were measured using h.p.l.c. [25] and compared with the specific radioactivity of PtdIns(4,5)P_2. No significant differences were found between the specific radioactivity of ATP γ-phosphate group and the specific radioactivities of the labelled phosphates in the polyphosphoinositides.

Incubation of erythrocytes

Unless otherwise stated, labelled cells at a haematocrit of 20% were incubated at 37 °C in Hepes-saline and treated with MC540, DMPC, Ca^{2+}/A23187 or deoxyglucose/iodoacetamide as detailed below. After incubation the cells were sedimented (500 ***g***, 5 min) and AChE activity, phospholipid release and extent of lysis were measured in the supernatant. Values of 100% AChE activity, phospholipid release and lysis were calculated from a sample taken from the incubation mixture at the beginning of the experiment. Microvesicles were sedimented from the supernatant by centrifugation at 30000 ***g*** for 30 min (except for MC540 vesicles, which were sedimented at 100000 ***g*** for 1 h). Microvesicles from stored blood were washed by centrifugation several times to remove any contaminating ghosts. SDS/PAGE, t.l.c. and phosphorus analysis were carried out on the vesicles and the ghosts from treated cells.

MC540 treatment. Cells at 5% haematocrit were treated with 62.5 μM-MC540 (added as a 10 mM solution in dimethyl sulphoxide) for 2 min. The incubation was terminated by cooling in ice for 5 min.

DMPC treatment. A suspension of DMPC was prepared by sonicating the dry lipid in the incubation buffer (0.5 mg/ml) using an MSE sonicator (14 μm peak-to-peak amplitude). Cells were incubated for 2.5 h in this suspension as described previously [7]. Because the microvesicle sample was contaminated with DMPC, total microvesicle phospholipid release was calculated by measuring phosphatidylethanolamine and phosphatidylserine after separating the lipids by t.l.c. (assuming that these phospholipids combined represent 40% of total lipid [26]).

Calcium ionophore treatment. Cells were incubated with 1 mM-$CaCl_2$ and 5 μM-A23187 for 25 min as previously described [27].

ATP depletion. In order to achieve ATP depletion, cells were incubated with 5 mM-iodoacetamide and/or 5 mM-deoxyglucose for 24 h [28].

Aging *in vitro*. Whole blood was stored for 10–15 weeks in citric acid/sodium citrate/dextrose at 4 °C.

RESULTS

When erythrocytes are incubated with MC540 or DMPC, with Ca^{2+}/A23187, under conditions of energy depletion, or after storage for several weeks, the cells change their morphology from discocyte through echinocyte to spherocyte and release microvesicles into the medium [1,5,7,9,27]. As shown previously [6,9,29], AChE release strongly correlates with phospholipid release.

Membrane protein composition

Based on results from SDS/PAGE, microvesicles released under any of the conditions employed clearly contained only band 3 and glycophorin in addition to cytosolic proteins [5,9,30]. However, the amounts of band 3 and glycophorin relative to phospholipid in microvesicles were substantially less than in either the original cells or the residual treated cells. Thus for MC540, DMPC, Ca^{2+}/A23187 or storage microvesicles, the amounts of band 3 and glycophorin were between 40 and 50% of the values observed in ghosts from untreated cells (Table 1). Higher values (60–70%) were seen in microvesicles from ATP-depleted cells, and this result was the same whether iodoacetamide or deoxyglucose was used for ATP depletion, although iodoacetamide gave a somewhat higher microvesicle yield. A substantially lower value (20%) for band 3, but not for glycophorin, was observed in the case of microvesicles from stored cells.

In contrast with the relative depletion of band 3 and glycophorin, a 2–3-fold enrichment of AChE relative to phospholipid was observed in the microvesicle preparations, similar to that described previously [29,31]. Total activity of this enzyme was not changed, since there was an equivalent decrease in activity in the residual cells [32].

Membrane lipid composition

The overall phospholipid composition of the microvesicles, the parent erythrocytes and the MC540-treated erythrocytes was very similar, with little variation in the relative amounts of the

Table 1. Segregation of band 3 and glycophorin into microvesicles

The ratio of band 3 and glycophorin concentration (derived from gel scans) to phospholipid was measured for control ghosts from untreated cells, and ghosts and microvesicles from cells treated with MC540, DMPC, Ca^{2+}/A23187 or iodoacetamide/deoxyglucose (ATP depletion) and from cells aged by storage. Values for the control ghosts are taken as 100%. Results are expressed as means ± standard error taken from four experiments (seven in the case of MC540) carried out in duplicate. The relative amounts of band 3 and glycophorin in the microvesicles from the different treatments were compared using analysis of variance. * Microvesicles from ATP-depleted cells contained significantly more band 3 and glycophorin than the other microvesicles ($P < 0.01$) and microvesicles from aged cells contained less band 3 than those from the other treatments ($P < 0.01$).

	Relative amount (%)			
	Band 3		Glycophorin	
Treatment	Ghosts	Microvesicles	Ghosts	Microvesicles
MC540	91±3	36±6	110±4	47±5
DMPC	108±7	41±4	99±8	51±4
Ca^{2+}/A23187	94±8	42±4	92±8	43±6
ATP depletion	124±9	61±3*	114±8	71±4*
Storage	99±7	19±1*	113±7	41±4

Table 2. Content of PtdIns(4,5)P_2 and PtdIns(4)P in ghosts from treated cells and microvesicles

Phospholipids were extracted and analysed as described in the text. The amounts of PtdIns(4,5)P_2 and PtdIns(4)P in the ghosts and microvesicles from cells treated with MC540, DMPC, Ca^{2+}/A23187 or iodoacetamide/deoxyglucose (ATP depletion) were measured from the radioactive counts and phosphate analysis, taking the values for the control ghosts as 100%. The results represent mol% of total phospholipid and are shown as means ± standard error from four experiments (seven for MC540) done in duplicate. Significant differences between treated cells and vesicles, by paired t tests, are indicated as follows: $*P < 0.05$; $**P < 0.02$; $***P < 0.01$; $****P < 0.001$.

	Content (mol%)			
	PtdIns(4,5)P_2		PtdIns(4)P	
Treatment	Ghosts	Microvesicles	Ghosts	Microvesicles
MC540	100±4	57±4****	90±3	56±3****
DMPC	114±9	55±5***	92±10	71±9
Ca^{2+}/A23187	55±7	35±5**	53±8	46±6
ATP depletion	23±2	16±3***	100±4	67±10*

major lipids [32]. Similar results were obtained for microvesicles obtained by the other treatments (results not shown).

The amounts of PtdIns(4,5)P_2 and PtdIns(4)P in the ghost samples from untreated cells were found to be approx. 1.6 mol% and 1.2 mol% respectively of total phospholipid (average of seven experiments done in duplicate). This compares well with values of approx. 1.5% and 0.9% respectively given by other workers [33–35]. However, the levels of these lipids, particularly PtdIns(4,5)P_2, were markedly reduced in the microvesicles from MC540 or DMPC treatment compared with the original cells (Table 2). The level of PtdIns remained constant at 1% in ghosts and microvesicles, so that the reduction in the polyphosphoinositides was not due to their dephosphorylation. Neither was there any evidence for breakdown of these lipids by a phospholipase C, since there was no increase in either diacylglycerol or phosphatidate in the ghosts or microvesicles (except with calcium treatment [27]). A very similar decrease in the content of the polyphosphoinositides was observed in microvesicles from cells subjected to ATP depletion or treatment with Ca^{2+}/A23187, although in these two cases the amount of PtdIns(4,5)P_2 in the treated cells was considerably reduced, due either to dephosphorylation of this lipid as a result of ATP depletion or, in the case of Ca^{2+}/A23187 treatment, to the activation of polyphosphoinositide phosphodiesterase by increased intracellular Ca^{2+} [36].

DISCUSSION

As we have shown recently [9], human erythrocytes undergo echinocytosis and microvesiculation when exposed to MC540. This compound seems to act in a similar way to other amphipathic molecules which partition into the outer membrane leaflet but which do not cross the cell membrane, such as DMPC [7] or glycocholate [8]. Microvesiculation appears to be an extreme consequence of intercalation of these amphipathic molecules selectively into the outer leaflet of the lipid bilayer. This induces localized curvature of the membrane, as predicted by the Sheetz & Singer bilayer couple hypothesis [37]. Very similar echinocytosis and release of microvesicles is seen when cells are subjected to procedures which diminish the amount of the polyphosphoinositides on the inner lipid leaflet of the membrane bilayer, either as a consequence of phosphomonoesterase activity after ATP depletion [33] or resulting from phosphodiesterase activity stimulated by a rise in the intracellular concentration of Ca^{2+} [27]. Again, these effects can be rationalized in terms of the Sheetz & Singer hypothesis.

The advantage of using MC540 for studies of microvesiculation is that no metabolic changes are involved and the effect is rapid; maximum release of microvesicles is seen in 2 min. This contrasts with the 24 h preincubation with metabolic inhibitors required in order to obtain microvesicles from ATP-depleted cells and with the complex biochemical changes which result from elevation of intracellular Ca^{2+} [27]. Even with DMPC (which does not produce metabolic changes), the lag-time before onset of vesiculation was approx. 1 h [7]. The most likely explanation for the difference in the speed of the effects of MC540 and DMPC is that the entry into the membrane outer leaflet depends on the concentration of free amphipath in the medium and that this in turn is controlled by the critical micellar concentration, which is relatively high for MC540 compared with DMPC (C. Hagelberg & D. Allan, unpublished work). Although Ott *et al.* [7] showed that DMPC intercalates into the bilayer within minutes, it may take an hour for a sufficient concentration to build up in the membrane outer leaflet to allow microvesicle release to occur.

Segregation of proteins into microvesicles

One notable feature of our results is that the content of band 3 relative to that of phospholipid in the microvesicles is only about 40% of that in the original cells or in the residual treated cells. This is consistent with the independent evidence that about 60% of the band 3 protein is bound to the cytoskeletal network via ankyrin or via glycophorin and band 4.1 [38,39], and that accordingly this fraction of band 3 is unable to diffuse into the microvesicles. Glycophorin A is decreased in the microvesicles to a similar extent as band 3, suggesting a comparable degree of skeletal attachment. It is possible that the similar behaviour of glycophorin and band 3 is due to a complex formation between these proteins [40,41].

In contrast with these findings, and as observed previously for microvesicles prepared by other procedures, there was a 2–3-fold increase in AChE specific activity in the microvesicles, although the total activity of this enzyme was not changed. AChE is one of a number of cell surface enzymes which appear to be attached by linkage to a modified phosphatidylinositol [42], so that it might have been expected that such enzymes would share in the rapid diffusion characteristic of lipids. A possible explanation for the markedly increased activity of AChE in the microvesicles is that the removal from the bilayer of some integral proteins which remain attached to the membrane skeleton effectively allows more room in the microvesicle membrane for the accommodation of those proteins like AChE which can diffuse freely. Put another way, removal of half of band 3 and glycophorin leads to a decrease in the surface packing pressure in the microvesicle membrane, which favours the diffusion of AChE out of the more crowded membrane of the residual cells. It seems unlikely that the increased activity of AChE could be due to a greater surface curvature in the microvesicles, since the residual cells are very echinocytic (i.e. locally curved) but show a decrease in enzyme activity [32].

The patterns of protein segregation in the MC540-, DMPC- or Ca^{2+}-induced microvesicles were very similar (Table 2), suggesting that the effects of these agents reflected an underlying organization of the membrane. The only significant difference was with ATP depletion, where it appeared that somewhat more band 3 and glycophorin was free to diffuse into the microvesicles, perhaps suggesting that energy depletion affected the ability of these proteins to bind to the membrane skeleton. This effect does,

however, seem less marked than the results reported by Lutz *et al.* [5], who found essentially no decrease in the amounts of band 3 and glycophorin in vesicles released as a result of energy depletion, indicating no barrier to the free diffusion of these proteins. A substantially lower value for band 3 (20%) but not for glycophorin was observed in the case of microvesicles from stored cells, although here it is possible that degradation of band 3 occurred during the long period of storage.

Partitioning of polyphosphoinositides into microvesicles

The composition of the major phospholipids in the microvesicles prepared by MC540 treatment [32] or any of the other procedures was very similar to that of the original cells. This is to be expected, since phospholipids generally are thought to diffuse freely in the plane of the membrane, and thus the microvesicles should contain a representative sample of these lipids. The only phospholipids to behave differently were the polyphosphoinositides: the microvesicles only contained about half as much of the polyphosphoinositides as the original cells (compared on the basis of total phospholipid content), suggesting that only about half of these lipids are free to diffuse. This inference would be consistent with the idea that the polyphosphoinositides may bind to cytoskeletal proteins and perhaps mediate interactions between band 4.1 and glycophorin [14].

Studies using ^{31}P-n.m.r. have indicated that four molecules of inositol phospholipid are tightly bound to each glycophorin molecule [43]. If it is assumed that the most likely lipid to be associated with glycophorin is PtdIns(4,5)P_2, these n.m.r. studies could account for a substantial proportion of the non-mobile pool of PtdIns(4,5)P_2. Thus there are approx. 2.5×10^8 molecules of phospholipid in each cell, and if PtdIns(4,5)P_2 accounts for 1.5% of this, then there are 3.8×10^6 molecules of PtdIns(4,5)P_2 per cell. Since 4×10^5 molecules of glycophorin are present in each cell [13], 1.6×10^6 molecules of PtdIns(4,5)P_2 per cell (only 42% of the total) are calculated to be bound to glycophorin. There is, therefore, too much PtdIns(4,5)P_2 to explain its distribution between cells and microvesicles solely in terms of binding to glycophorin. However, it may be more than coincidence that such similar proportions of band 3, glycophorin and PtdIns(4,5)P_2 are able to diffuse into the microvesicles; perhaps this is a reflection of ternary interactions between these proteins, PtdIns(4,5)P_2 and the membrane skeleton.

This restricted mobility of the polyphosphoinositides in the membrane through the interaction with proteins might be expected to be reflected in metabolic pooling of these lipids. Thus it would not be surprising if accessibility of polyphosphoinositides to phosphatases, kinase and the Ca^{2+}-dependent phosphodiesterase was different for protein-bound inositide compared with freely diffusible inositol lipid. Such considerations could explain the observations of King *et al.* [44], who found that the maximum specific radioactivity of PtdIns(4,5)P_2 was only 30% of the specific radioactivity of the γ-phosphate of ATP, suggesting that a large part of the polyphosphoinositide pool was not metabolically accessible. However, after the long period of incubation with ^{32}P that we used, the polyphosphoinositides were labelled to equilibrium with ATP (C. Hagelberg & D. Allan, unpublished work) so that we did not find any differences between the specific radioactivities of polyphosphoinositide which partitioned into microvesicles and that which did not.

This work was funded by the Medical Research Council.

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Received 9 July 1990/23 August 1990; accepted 28 August 1990