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ON THE MECHANISM OF VESICLE RELEASE FROM ATP-DEPLETED HUMAN RED BLOOD CELLS

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The release of spectrin-free vesicles from ATP-depleted human red blood cells (Lutz et al. (1977) J. Cell. Biol. 73, 548) can be considered the final step of a shape change from discocytes to echinocytes. The study of physical and chemical properties of released vesicles suggests that vesicle release is not merely a consequence of charge alterations within either monolayer of the budding membrane. Fresh membranes and released vesicles have within experimental error the same sialic acid content per surface area and the same electrophoretic mobilities. Vesicle release cannot be stimulated by doubling the charge density on the outer monolayer by means of a phospholipase D-treatment, but correlates with a breakdown of polyphosphoinositides to diacylglycerol on the inner monolayer. This breakdown does not lead to a significant change in the negative charge density on the inner monolayer, because an increased phosphatidate content compensates for this alteration. Furthermore, polyphosphoinositide breakdown and diacylglycerol production are not the rate-limiting step in vesicle release from ATP-depleting red blood cells. This is evident from the fact that 10 mM EDTA inhibits vesicle release to 75% without affecting polyphosphoinositide breakdown and diacylglycerol production. Hence, diacylglycerol formation may be sufficient for membrane budding as suggested earlier (Allan et al. (1976) Nature 261, 58), but vesicle release requires a second, as yet unidentified process.

Introduction

The shape change from discoid human red blood cells to echinocytes induced by ATP depletion is reversible at the onset [1]. It becomes irreversible when echinocytic blebs start to bud off as spectrin-free vesicles [2]. Consequently, vesicle release can be considered the final step in membrane evagination [3].

In an attempt to explain membrane budding the effects of cationic and anionic anesthetics on erythrocyte shape change were studied and led to the formulation of the bilayer couple hypothesis [4–6]. In its most general form the hypothesis predicts that the two halves of the lipid bilayer, while remaining coupled to one another, respond independently to various perturbations. Thus, one-half of the bilayer may expand relative to the other in response to insertion of a small number of components such as drugs, diacylglycerol or negatively charged phospholipids. The expansion of one-half of the bilayer would lead to a curvature of the entire membrane. However, it was recently shown that amphipatic drugs do not partition into biological membranes as assumed in the bilayer couple hypothesis [7]. Yet direct evidence in favor of this hypothesis comes from studies on the release of spectrin-free vesicles from erythrocytes treated with ionophore and calcium [8]. Evidence suggested that a calcium-activated phosphodiesterase produces diacylglycerol from negatively charged phospholipids on the inside of the bilayer [9]. The two processes, the decrease in negative charge density and the production of diacylglycerol, could promote budding in an additive manner: (a) diacylglycerol as a

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Abbreviations. SDS, sodium dodecyl sulfate: PMSF, phenylmethylsulfonyl fluoride, EGTA, ethyleneglycol bis(amino-ethyl ether)-*N,N'*-tetra-acetic acid.

diffusable intercalating molecule could equally partition between both monolyers and thus reduce the lateral pressure of the inner monolayer while expanding the outer [10]; (b) the reduction of the negative charge density on the inner monolayer could result in a decreased lateral pressure within this monolayer as a consequence of reduced charge repulsion in regions tending to bud outwards [11–13]. Little evidence is at hand to evaluate whether both charge density change and/or production of a diffusable intercalating molecule are required for membrane budding and vesicle release.

In this report we studied some chemical and physical properties of both monolayers of vesicles released from ATP-depleted erythrocytes. If chemical and physical alterations occur as part of the budding mechanism, they persist in released vesicles and their analysis allows us to get some insight into the requirements of membrane evagination. Based on our findings, evagination on ATP-depleting erythrocytes occurs without a significant charge density change on either side of the membrane, but could be induced by diacylglycerol. However, an additional yet unidentified process is involved in the mechanism of vesicle release, because vesiculation can be inhibited without affecting the polyphosphoinositide breakdown to diacylglycerol.

Materials and Methods

Whole human blood (O, Rh^+) was obtained from the Swiss Red Cross, Zurich, and collected in citrate-phosphate dextrose. Cells washed once were passed through a cotton column to reduce white cell contaminations as described previously [14]. The eluted cells were washed three times in the same buffer. Washed cells were ATP-depleted by either method 1, following a modified procedure as published previously [2], or by method 2 according to Ref. 15 in the presence of calcium and ionophore A23187.

Method 1. Washed and packed human red blood cells were added to 4 vol. of a medium as given in Ref. 2, having an osmolarity of 310 mosM and a pH of 7.5. This cell suspension was placed into dialysis bags. The bags were suspended in a container filled with an excess of medium and incubated in a water bath at 37°C under gentle agitation [14]. The pH in the medium surrounding the bags was ajusted to 7.4 after 6 and 15 h. After incubation for 40 h the content of the bags was centrifuged for 7 min at 2000 rev./min in a Sorvall SS34 rotor. The supernate was collected and the pellet washed once with a buffer containing 150 mM NaCl/5 mM $NaKHPO_4$ (pH 7.4). Both supernatants were combined, diluted into buffer, and filtered through a Sartorius membrane filter with a pore size of 0.8 μm to remove residual cells. The filtrate was centrifuged at 9000 rev./min for 50 min in a Sorvall GSA rotor. The pellet was resuspended in a small volume of either 150 mM NaCl (for the lipid extractions) or in 150 mM NaCl/5 mM $NaKHPO_4$ (pH 7.4) (for electrophoretic mobility measurements and other determinations).

Method 2. Treatment with ionophore A23187 (a gift from Eli Lilly Laboratories, Indianapolis, IN, U.S.A.) and calcium: these experiments were carried out according to Allan and Michell [15]. Vesicles were collected by the same procedure as described for method 1.

Enzyme treatments of cells and vesicles

(a) Pretreatment of cells with phospholipase D. Phospholipase D was obtained from Sigma (from cabbage, type I). Washed cells were treated with either 0, 4 or 8 mg phospholipase D/50 ml packed cells for 1–2 h at 50% hematocrit in an isotonic medium containing 50 mM glycylglycine/5 mM KCl/103 mM NaCl/5 mM $NaKHPO_4$/0.6 mM adenine/12 mM inosine/2 g/l glucose/300 mg/l streptomycin/250 mg/l penicillin/20 mg/l PMSF (pH 7.4). $CaCl_2$ (2 mM) was added to start the reaction. The enzyme reaction was blocked by adding 2 mM EGTA to complex Ca^{2+}. Then cells were twice washed isotonically.

(b) Treatment of spectrin-free vesicles with immobilized neuraminidase. Neuraminidase (from *Vibrio cholerae,* Behring Institut, F.R.G.) was coupled to CNBr-activated Sepharose 6-MB (Pharmacia, Sweden) by the coupling procedure given by Pharmacia resulting in immobilized enzyme that cleaved 1 μmol sialic acid from human red cell membranes, in 30 min, at room temperature and pH 6.5/ml packed gel. Vesicle suspension (0.5 ml) was mixed with packed gel (0.5 ml) of immobilized neuraminidase in a buffer containing 150 mM NaCl/5 mM $NaKHPO_4$/2 mM $CaCl_2$ (pH 6.5). The mixture was rotated end-over-end for up to 4 h at room temperature. For separation of gel and vesicles, the mixture was allowed to

stand for some minutes. Samples of the supernate were used for electrophoretic mobility measurements, sialic acid determinations and SDS-polyacrylamide gel electrophoresis.

Chemical analysis

Protein in isolated membranes was measured by standard techniques. In vesicles, the content of band 3 protein was determined from bound Coomassie blue on a SDS-polyacrylamide gel by scanning the dried gel on a gel scanner (Integraph, Bender and Hobein, Zurich). This relative measure was referred to that obtained for 15 μg hemoglobin-free membranes. The sialic acid content in membranes was determined by the procedure given in Ref. 16. Sialic acid content in vesicles was obtained as follows. vesicles were digested with 0.1 N H_2SO_4 at 80°C for 1 h and samples were adjusted to pH 7.0. These samples were extracted with an equal amount of cyclohexanon to remove the red colour originating from hemoglobin, which interferes with the assay. This treatment shifted the red coloured material into the organic phase, while the sialic acid remained in the water phase. Samples of the water phase were analyzed according to Warren [16].

Neutral and acidic lipid extractions were combined as follows. 2.5 ml packed membranes or 2.5 ml vesicle suspension were homogenized with 15 vol. $CHCl_3/CH_3OH$ (1 · 1, v/v) and 150 μmol $CaCl_2$ by stirring at room temperature for 20 min. The mixture was then centrifuged for 4 min at $3000 \times g$. The clear supernate was transferred to a separation funnel and the extraction procedure was repeated twice with 3 vol. $CHCl_3/CH_3OH$ (2 1, v/v). The clear supernates were combined with the first and 0.2 vol. 0.88% KCl were added, shaken and allowed to stand overnight at 4°C. The clear organic phase was then evaporated and the neutral lipids were dissolved in a small amount of $CHCl_3/CH_3OH$ (2 . 1, v/v). The subsequent extraction of the acidic lipids was performed with the pellet remaining after extraction of neutral lipids. This procedure was carried out according to Hauser et al. [17].

The two-dimensional chromatography of neutral phospholipids was carried out on thin-layer plates silica gel H, thickness 0.25 mm (Anachem Ltd., U.K.) [18]. About 50 μg phosphorus were applied to the plates. Chromatography of the acidic phospholipids was carried out according to Gonzalez-Sastre and Folch [19]. Standard solutions of L-α-phosphatidylinositol 4,5-diphosphate, L-α-phosphatidylinositol, sphingomyelin (Type I) and phosphatidylcholine (egg lecithin, grade I, Lipid products, South Nutfield) were used to identify the spots on the thin-layer plates run with acidic phospholipids.

Dried samples originating from lipid extracts or silica gel spots scratched out from thin-layer plates were digested in glass test tubes. Phosphorus was determined according to Chen et al. [20].

Diacylglycerol was isolated on silica gel H thin-layer plates, thickness 0.25 mm (Anachem Ltd., U.K.) according to Freeman and West [21]. The spots identified as diacylglycerol were scratched out and determined according to Amenta [22]. 1,2-Diolein (Sigma) served as a standard. Aliquots of this standard solution were chromatographically separated and determined under the same conditions as the samples. The unknown diacylglycerol content in samples was calculated using the equation given in Ref. 22.

Physical analysis

Electrophoretic mobilities of isolated vesicles were determined according to Hauser et al. [11] with a commercially available particle electrophoresis apparatus (Rank Brothers, Cambridge, U.K.) at 25°C in a buffer system containing 150 mM NaCl/5 mM $NaKHPO_4$, at pH 7.4.

SDS-polyacrylamide slab gels were performed as described previously [23]. Gels were stained for proteins with Coomassie blue in 50% methanol/10% acetic acid.

Results

Chemical and physical properties of the outer monolayer of spectrin-free vesicles released from ATP-depleted human red blood cells

ATP-depletion of human red blood cells induces a shape change from discocytes to echinocytes [1]. Echinocytes release some of their blebs as spectrin-free vesicles irrespective of whether the cells are rapidly ATP-depleted, by increasing the cytosolic calcium concentration [8], or slowly ATP-depleted by incubation in a glucose-free medium [2]. We demonstrate here that the chemical and physical

properties of the outer monolayer, sialic acid content and electrophoretic mobility, remain unaltered during budding of the membrane and release of spectrin-free vesicles induced by both types of ATP-depletion.

The electrophoretic mobilities of both types of vesicle are statistically the same (Table I) and compare well with that of intact cells. This result is substantiated by the findings that both types of vesicles have, within experimental error, the same content of sialic acid per band 3 protein as intact membranes (vesicles method 1: −7.3 ± 4%, vesicles method 2: −3.2 ± 8%) (Table I).

On the other hand, the phosphatidate content of vesicles is increased as compared to that of intact membranes. The phosphatidate content of vesicles released from calcium-treated cells is twice as high as in those obtained by nutrient deprivation (Table I). The differing phosphatidate content does not involve the outer monolayer as is evident from the same electrophoretic mobilities (Table I) and the residual electrophoretic mobility of desialylated vesicles (Fig. 1). Desialylation was carried out with immobilized neuraminidase to avoid adsorption of the positively charged enzyme [26]. The residual electrophoretic mobilities of both types of vesicles – as obtained by extrapolation to zero sialic acid content of the curves in Fig. 1 – are the same and compare well with that estimated for intact cells (data from Ref. 24). Since the protein composition of both types of spectrin-free vesicles is also comparable as judged from SDS polyacrylamide gels, we conclude that both composition and negative charge density on the outer monolayer of the membrane remain virtually unaltered during budding and vesiculation of red blood cells.

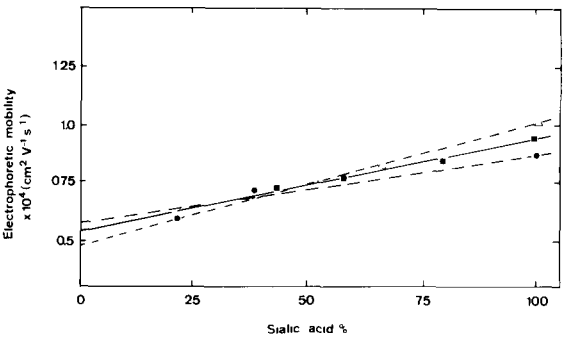

Fig. 1. Electrophoretic mobility of spectrin-free vesicles upon treatment with immobilized neuraminidase The assay conditions were described under Materials and Methods. Percentages of sialic acid were determined from the content of sialic acid (μg sialic acid per mg band 3 protein) and were referred to that present in vesicles not treated with neuraminidase. ■——■, Spectrin-free vesicles isolated by method 1 ●——●, Spectrin-free vesicles isolated by method 2. △, Intact cells (data from Ref 24).

Contrary to these findings, evagination and invagination observed on intact cells treated with phospholipase C and particularly phospholipase D appear dependent on charge density alterations induced by these enzymes [27]. Hence, we tested whether vesicle release is promoted by an artificially increased negative charge density on the outer monolayer. Increased charge density on eryhrocytes was obtained by treatment of intact cells with phospholipase D. This treatment results in the formation of

TABLE I

CHARACTERIZATION OF SPECTRIN-FREE VESICLES RELEASED FROM ATP-DEPLETED HUMAN RED BLOOD CELLS

Sialic acid, band 3 protein and phosphatidate contents were determined as given in Materials and Methods. The electrophoretic mobility of vesicles was determined from three to five independent experiments. For each experiment at least 20 measurements were averaged. All values are listed with standard errors.

Type of membrane	μg sialic acid/mg band 3 protein	Electrophoretic mobility X 10^4 ($cm^2 \cdot V^{-1} \cdot s^{-1}$)	Phosphatidate in % of the total phospholipid content
Vesicles isolated by method 1	69.7 ± 3.1 (n = 4)	−1.03 ± 0.13 (n = 5)	2.44 ± 0.2 (n = 5)
Vesicles isolated by method 2	72 9 ± 5 7 (n = 3)	−0.99 ± 0.11 (n = 3)	4.7 ± 0.1 (n = 3)
Membranes isolated from fresh cells	75.2 ± 2.2 (n = 3)	−1.08 *	1.4 ± 0.3 (n = 3)

* Values from Ref. 24 and 25.

phosphatidate exclusively on the outer monolayer, because the membrane is impermeable to phospholipases [28]. If charge density alterations were a rate-limiting step in evagination and vesiculation, the enzymatically-produced phosphatidate should stimulate echinocytosis and vesicle release during ATP-depletion by an enhanced charge repulsion effect.

Untreated as well as phospholipase D-treated cells were ATP-depleted by either technique. The isolated spectrin-free vesicles released from phospholipase D-treated cells contain increased amounts of phosphatidate and had a significantly higher electrophoretic mobility than those from untreated cells (Fig. 2). In fact, the electrophoretic mobility of vesicles released from phospholipase D-treated cells increases linearly by the same increment for both types of vesicles with increasing total phosphatidate content. Furthermore, this increase in electrophoretic mobility obtained for vesicles from phospholipase D-treated cells is preserved upon treatment with neuraminidase (Fig. 3). Therefore, the increased electrophoretic mobility is very likely to be due to phosphatidate localized on the outer monolayer (Fig. 3). Even though the phosphatidate content in the outer monolayer was raised up to 2.5% of the phospholipids present, this doubling of the charge density did not influence vesicle release at the pH used for the electrophoretic mobility measurements (Fig. 4). The yield of vesicles remains virtually unaltered, irrespective of whether vesicles are released from phospholipase D-pretreated cells or from untreated cells. The protein pattern of vesicles released from untreated as well as from phospholipase D-pretreated cells, as detected on SDS-polyacrylamide gels, remains unaltered (data not shown). These results suggest that the last step in echinocytosis, the release of vesicles, is largely independent of the surface charge density of red blood cells.

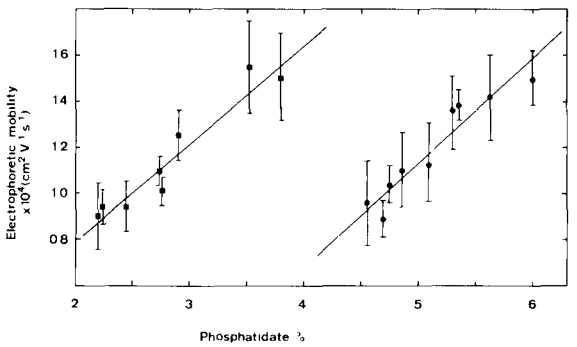

Fig. 2. Electrophoretic mobility of spectrin-free vesicles as a function of their total phosphatidate content. Electrophoretic mobility and phosphatidate content were measured as described under Materials and Methods. Electrophoretic mobility measurements are the mean of at least 20 determinations with standard errors given Both types of vesicles were released from ATP-depleted cells that were pretreated with phospholipase D. Electrophoretic mobility of spectrin-free vesicles isolated according to method 1 (■) and method 2 (●)

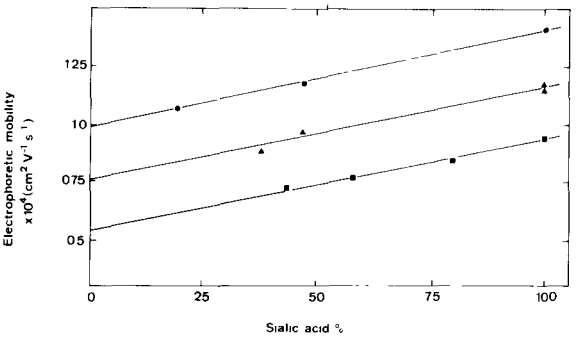

Fig. 3. Electrophoretic mobility of spectrin-free vesicles upon treatment with immobilized neuraminidase isolated from phospholipase D pretreated cells. The assay conditions were the same as in Fig. 1. Vesicles isolated from phospholipase D-pretreated cells, but not desialylated, had within an error of ±3.8% the same sialic acid content as vesicles from phospholipase D-untreated cells. Vesicles were treated with neuraminidase as given in Materials and Methods. ■———■ Spectrin-free vesicles isolated by method 1 from cells not treated with phospholipase D. Spectrin-free vesicles isolated by method 1 containing increased phosphatidate contents following phospholipase D treatment (▲, 2.75%, ●, 3.4% phosphatidate of total phospholipids).

Chemical properties of the inner monolayer of ATP-depleted human red blood cells and of spectrin-free vesicles released in the absence of free calcium

Membranes of ATP-depleted red blood cells and released vesicles obtained with ionophore and Ca^{2+} have a significantly reduced content of polyphosphoinositides, which appears to be due to a calcium-activated endogenous phosphodiesterase activity [30]. The breakdown of these negatively-charged phospholipids was shown to correlate with diacylglycerol production and membrane budding [12]. Therefore, we analyzed whether breakdown of polyphosphoinositides also occurs during vesiculation induced by

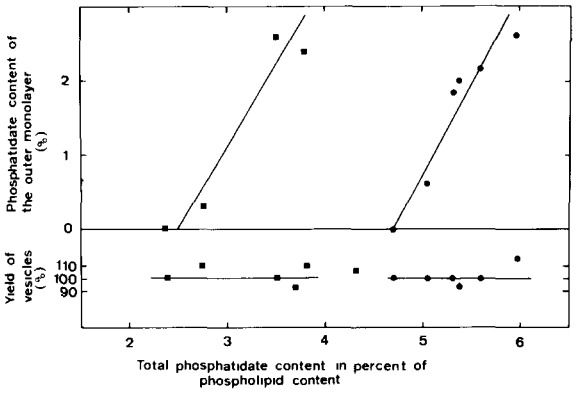

Fig 4. Yield of vesicles and phosphatidate content of the outer monolayer as a function of the total phosphatidate content following treatment of cells with phospholipase D. Phospholipase D-untreated and -treated cells were subjected to ATP-depletion for 40 h (see Materials and Methods). At the end of the incubation time amounts of released vesicles and their phosphatidate contents were determined. The lowest phosphatidate values shown (mean of four determinations) originate from both types of vesicles released from cells that were not pretreated with phospholipase D. The phosphatidate content in the outer monolayer was calculated from the electrophoretic mobility of released vesicles. This electrophoretic mobility increases linearly with increasing total phosphatidate content (Fig. 2) by an increment of $-0.4 \cdot 10^4$ ($cm^2 \cdot V^{-1} \cdot s^{-1}$) per 1% phosphatidate. The same increment was also reported by Hauser et al. [29] The difference in electrophoretic mobilities of vesicles from treated and untreated cells was divided by the increment as given above. Assuming that no phosphatidate is present in the outer monolayer of vesicles from untreated cells, this value represents the phosphatidate content of the outer monolayer in percent of the total phospholipids. By myltiplying this value by 2, percent phosphatidate per lipids in the outer monolayer was obtained. The yield of vesicles was determined from the amount of band 3 protein, which served as a surface marker in the vesicles. Its content was determined from the amount of bound Coomassie blue on a 8% polyacrylamide gel. The value obtained for cells not pretreated with phospholipase D was set to 100%. Vesicles obtained by method 1 (▪) or method 2 (•).

metabolic ATP-depletion in the absence of added calcium or in the presence of EDTA.

Fig. 5a shows that membranes from metabolically ATP-depleted cells (M_0) have a decreased content of polyphosphoinositides but a slightly elevated content of diacylglycerol as compared to membranes from fresh cells (Fig. 5a, M). EDTA concentrations ranging from 0.1 to 10 mM added to ATP-depleting cells inhibit neither the breakdown of these negatively-charged phospholipids nor the formation of diacylglycerol. (Fig. 5a, M_1, M_{10}).

Hence, breakdown of polyphosphoinositides to diacylglycerol on the inner monolayer is not dependent on an influx of Ca^{2+}. The breakdown occurs even in the presence of up to 10 mM EDTA. The same conclusions can be drawn from the contents of polyphosphoinositides and diacylglycerol in released vesicles (Fig. 5b). Vesicles contain half the amount of polyphosphoinositides found in fresh membranes (Fig. 5b, Vo). In vesicles released from cells that were ATP-depleted in 10 mM EDTA the diacylglycerol content reached $1 \pm 0.06\%$ of the total phospholipids, while that of polyphosphoinositides was greatly reduced. The breakdown of polyphosphoinositides and the concomitant diacylglycerol production are evident both in membranes of ATP-depleted cells and in released vesicles and are not inhibited by EDTA. Furthermore, this breakdown seems independent of the free Ca^{2+} concentration within the cell (less than 0.1 μM, Ref. 31). The reason for this tentative conclusion is that EDTA seems to penetrate cell membranes, as shown by adding ^{14}C-labeled EDTA to ATP-depleting red blood cells. After ATP-depletion, the amount of total EDTA trapped and taken up in washed and packed cells was 7.2 pmol, 59 pmol and 495 pmol/100 μl cells at 0.1 1.0 and 10 mM EDTA, respectively. Although these data were not corrected for adsorption, the linear increase with increasing EDTA concentration suggests a significant uptake of EDTA.

The breakdown of polyphosphoinositides results in a decrease of negatively-charged species on the inner monolayer. This loss of negative charges does not seem to alter the charge density significantly, because it is compensated by an increased phosphatidate content (see Table I). One parameter that clearly changes is the diacylglycerol content. However, the consideration of another aspect indicates that an increase in the diacylglycerol content is not the only alteration necessary for vesicle release.

If we analyze the extent of vesiculation rather than the composition of released vesicles as a function of increasing EDTA concentrations, we observe a drastic inhibition of vesicle release (Fig. 6). Above 1 mM EDTA vesiculation is inhibited, reaching 90% inhibition at 15 mM. As pointed out earlier, this inhibition cannot be due to a reduced formation of

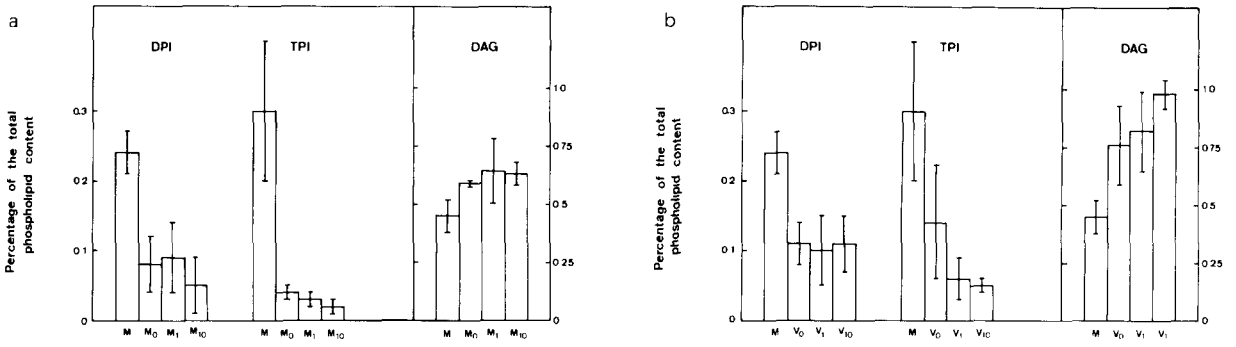

Fig 5. Diphosphoinositide, triphosphoinositide, and diacylglycerol content of ATP-depleted red blood cells (a) and spectrin-free vesicles (b). The values are the mean of at least three determinations with the standard errors given and represent the lipids recovered from thin-layer plates (recoveries 90% or more) Isolation and characterization are described under Materials and Methods. M. Cell membranes from freshly drawn blood. M_0,M_1,M_{10}· Membranes from cells that were ATP-depleted in a medium containing 0,1, or 10 mM EDTA, respectively. V_0, V_1, V_{10}: Vesicles released from cells that were ATP-depleted in a medium containing 0,1 or 10 mM EDTA, respectively. DPI, diphosphoinositide, TPI, triphosphoinositide; DAG, diacylglycerol.

diacylglycerol in the membrane of ATP-depleting cells. Thus, EDTA at high concentrations appears to interfere with an additional process required for vesicle release but not for polyphosphoinositide breakdown. Since the protein composition (Fig. 7) and surface properties of vesicles released at 10 mM EDTA remain the same as in the absence of EDTA, this finding is a strong indication of a second trigger for vesicle release, which is independent of diacylglycerol production.

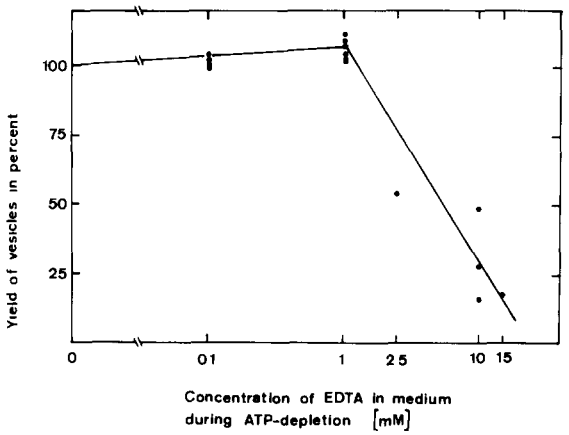

Fig. 6. Yield of spectrin-free vesicles released from ATP-depleted red blood cells in the presence of increasing EDTA concentrations. Cells were ATP-depleted according to method 1 in the presence of the EDTA concentrations given. The yield of vesicles was determined as given in Fig. 4

Discussion

This study of the composition of negatively-charged phospholipids in vesicles released from ATP-depleted human red blood cells demonstrates that membrane budding and vesiculation proceed without a significant change in the negative charge density on both monolayers of the membrane. Irrespective of whether cells are ATP-depleted metabolically or by addition of ionophore and Ca^{2+}, the isolated vesicles have the same electrophoretic mobilities and sialic acid contents. However, the vesicles have an elevated phosphatidate content as compared to that of fresh membranes.

The two types of vesicles differ in their phosphatidate content: it is higher in vesicles obtained from ionophore-treated cells than in those from metabolically-depleted cells. The reason for this difference is not known but may be related to the increased endogenous calcium concentration by ionophore treatment of intact cells. The increased amount of phosphatidate in both types of vesicles is located on the cytoplasmic side of the membrane as concluded from the unchanged electrophoretic mobility and sialic acid content. This finding suggests that a flip-flop of phosphatidate as observed in unilamellar liposomes with a half-time of 30–40 min [32] does not occur in intact cell membranes, though the phosphatidate distribution is highly asymmetric. The increased phosphatidate content on the inner

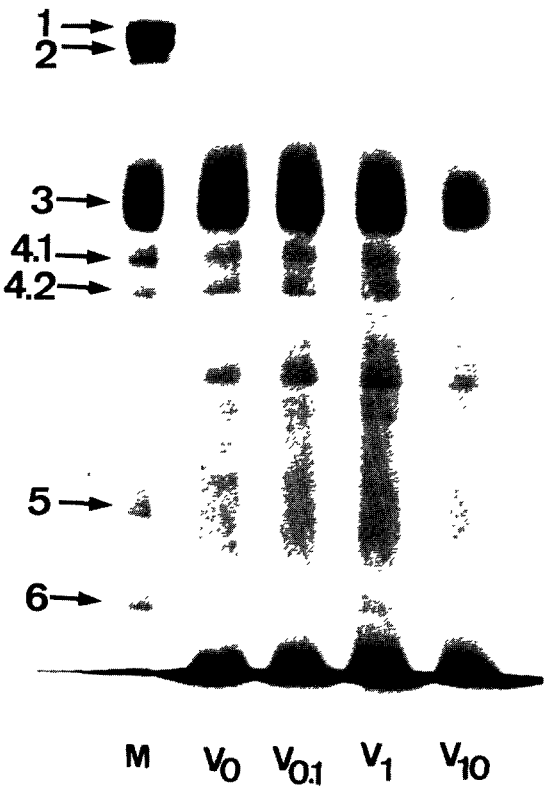

Fig. 7. Coomassie blue stained protein band pattern of an 8%-polyacrylamide gel from vesicles of red blood cells ATP-depleted in the presence of increasing EDTA concentrations. ATP-depletion and SDS-polyacrylamide gels were carried out as given in Materials and Methods Each sample applied to the gel contained 5 μg band 3 protein. M fresh cell membranes (15 μg protein). V_0, $V_{0.1}$, V_1, V_{10} Spectrin-free vesicles released from cells that were ATP-depleted in a medium containing 0,0 1,1 and 10 mM EDTA, respectively

monolayer compensates for the decrease in the negative charge density due to a breakdown of polyphosphoinositides (the content of phosphatidylserine does not change significantly). Thus, vesicle release occurs without a significant alteration of the charge density on either side of the membrane. Consequently, expansion of the outer monolayer during echinocytic budding and vesicle release has to be triggered by an uncharged molecule.

However, theoretically it should be possible to induce expansion of the outer monolayer by increasing the concentration of negatively-charged phospholipids that require a higher mean surface area than corresponding uncharged molecules [33]. Treatment of intact cells with phospholipase D, from cabbage, originally described as ineffective [34], acts on intact red blood cells by exposing negatively-charged phosphatidate. It doubles the charge density on the outer monolayer without increasing vesicle release.

While phospholipase D from *Streptomyces chromofuscus* had an effect on erythrocyte shape, but produced invagination rather than evagination, phospholipase D from cabbage did not induce any detectable shape change [27]. The obvious, though inverse, shape changes observed by Fujii and Tamura [27] occurred at phosphatidate concentrations exceeding those obtained in our experiments. We did not investigate whether similarly high phosphatidate concentrations would in fact alter vesicle release, because phospholipase D treatments were optimized so as to avoid any detectable hemolysis. Although this treatment was mild, it produced a substantial surface area change as estimated on the basis of phosphatidate surface areas determined in the Langmuir balance [33]. If we assume that the observed 2.5% phosphatidate were derived from 2.5% phosphatidylcholine, the expansion of the outer monolayer is about 1% at a lateral pressure of 5 dynes/cm and 0.2% at 30 dynes/cm. The surface area change at 5 dynes/cm is in the order of the expansion obtained by redistribution of 1% diacylglycerol to both monolayers that was shown to induce a drastic shape change of red blood cells [8]. Based on this comparison an increased phosphatidate content should enhance membrane evagination. However, the lateral pressure in biological membranes might be higher, reaching 30 dynes/cm [35].

The polyphosphoinositide content on the cytoplasmic side of red blood cell membranes drops considerably during ATP depletion and vesicle release. previously, Allan and Michell reported a decreased polyphosphoinositide content in cells treated with ionophore and calcium [9]. Allan et al. [8] suggested that during ATP-depletion and vesiculation polyphosphoinositides are converted into a purely hydrophobic lipid, diacylglycerol. We demonstrate a similar enhanced production of diacylglycerol and a preferential uptake into vesicles obtained from nutrient-deprived cells. The content of diacylglycerol as

determined here for vesicles is identical with that reported by Allan et al. for vesicles released upon treatment of cells with calcium and ionophore; the corresponding content in fresh membrane is 10-times higher than reported in Ref. 9. In calcium-loaded erythrocytes, diacylglycerol production appears to be mediated by a calcium-stimulated phosphodiesterase activity [9]. As shown here for nutrient-deprived cells, the breakdown of polyphosphoinositides and the concomitant production of diacylglycerol are not only independent of added calcium but also proceed in the presence of 10 mM EDTA.

While EDTA has no effect on the diacylglycerol production in membranes of ATP-depleting red blood cells and on the properties of released vesicles, it greatly inhibits vesicle release above 1 mM. This inhibition could imply that vesicle release is a two-step process. The first step would be a breakdown of polyphosphoinositides to diacylglycerol which promotes membrane budding as expected from the bilayer couple hypothesis. This step is not rate-limiting for vesicle release and occurs even in the absence of free calcium. The second step would be the actual release of spectrin-free vesicles, which is inhibitable by high EDTA concentrations. The apparent inhibition by EDTA suggests the involvement of bound cations, possibly of calmodulin [36]. The molcular component that is inhibitable by high EDTA concentrations remains to be characterized.

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