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Effect of membrane cholesterol on dimyristoylphosphatidylcholine-induced vesiculation of human red blood cells

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During incubation of intact human erythrocytes with sonicated dimyristoylphosphatidylcholine (DMPC) vesicles, the cells change their discoid morphology to form echinocytes and finally give rise to the release of membrane vesicles. In this process, the red cell membrane accumulates DMPC and loses up to 15% of its cholesterol. On the other hand, replacement of 25% of the endogenous phosphatidylcholine species by DMPC without affecting the cholesterol level of the erythrocytes can be achieved by incubation with DMPC/cholesterol (1:1, mol/mol) sonicated vesicles in the presence of the phosphatidylcholine-specific phospholipid-transfer protein from bovine liver. This replacement also gives rise to an echinocytic cell morphology, but no membrane vesiculation can be observed. However, the vesiculation process can as yet be initiated upon a subsequent decrease of the cholesterol level, by incubation of those modified cells in the presence of sonicated vesicles of pure egg phosphatidylcholine. Incubation of native erythrocytes with pure egg phosphatidylcholine vesicles, on the other hand, results in cholesterol depletion, but does neither induce the formation of echinocytes nor the release of membrane vesicles. Cellular ATP levels are not affected during these incubations. From these results, it can be concluded that a decrease in cholesterol content of the erythrocyte membrane is essential for the DMPC-induced vesiculation of those cells.

Introduction

Release of vesicles from human red blood cells can be observed under a variety of conditions. Either metabolic starvation of the erythrocytes [1], or an increase of their intracellular calcium levels [2] results in vesiculation. The process has been suggested to occur in vivo during ageing of the cells [3] and sickle cells have been shown to release microvesicles as a consequence of repeated deoxygenation and reoxygenation [4]. Similarly, incubation of human red blood cells with sonicated vesicles composed of either 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) [5] or 1,2-dilauroyl-*sn*-glycero-3-phosphocholine [6], results in rapid crenation of the cells which is followed by a release of membrane vesicles that bud-off from the intact cells [5]. In case of the DMPC-induced release of membrane vesicles, this process is known to occur without ATP-depletion [5].

On the other hand, membrane-penetrating agents, such as lysophospholipids and amphipathic drugs, are able to induce shape changes in

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human red blood cells as result of a perturbation of the lipid bilayer balance [7–11]. These shape changes lead to the formation of echinocytes (crenated cells) or stomatocytes, but no vesicle release has been reported. Furthermore, a partial replacement of native erythrocyte phosphatidylcholine-species by certain disaturated species results in the formation of echinocytes without membrane vesiculation [12]. This suggests that echinocyte formation is not a sufficient prerequisite for the vesiculation to occur and indicates that an additional process is of importance in the mechanism of vesicle release from red blood cells.

It is well established that the cholesterol content of the erythrocyte membrane can be decreased by incubation of the cells with sonicated phosphatidylcholine vesicles [13]. Hence, the difference between substances that act as crenators and the phospholipids that induce vesicle release may be ascribed to their different capabilities to remove cholesterol from the cell membranes. To confirm this hypothesis, experiments have been designed in which both processes – incorporation of DMPC into the red cell membrane and the decrease of cholesterol levels – have been strictly separated from one another. The results show that neither incorporation of DMPC into the red cell membrane nor cholesterol depletion are sufficient to induce vesiculation. Only the combination of both processes causes a release of vesicles.

Materials and Methods

Materials

Fresh human blood samples were obtained from healthy adult donors. Erythrocytes were separated from plasma by centrifugation at 3000 rpm for 15 min at 4°C and washed three times with 10 mM Tris-HCl (pH 7.4)/144 mM NaCl. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Berchtold (Berne, Switzerland), egg phosphatidylcholine from Koch-Light (Colnbrook, Bucks, U.K.) and phosphatidic acid from Lipid Products (South Nutfield, Redhill, U.K.). 1,2-Di[1-^{14}C]myristoylphosphatidylcholine and glycerol tri[9,10(n)-^{3}H]oleate were supplied by Amersham International (Amersham, U.K.). Penicilline was obtained from Novo Industri (Copenhagen, Denmark) and streptomycin sulfate from Grogg Pharmaceutical Products (Berne, Switzerland). Aquacide III (flake poly(ethylene glycol)) was purchased from Calbiochem (San Diego, U.S.A.). Boehringer Monotest ® for cholesterol determinations was obtained from Boehringer, Mannheim, F.R.G., and ATP-monitoring reagent from LKB Wallac (Turku, Finland). All other reagents used were commercial products of analytical grade and obtained either from Fluka AG (Buchs, Switzerland) or from Merck (Darmstadt, F.R.G.).

Methods

Acetylcholinesterase activities were determined according to Ellman et al. [14]. The ATP content of red cell suspensions was measured using the bioluminescence fire-fly assay from LKB Wallac. Briefly, the red cell suspension was adjusted to a hematocrit of approx. 4% and protein was precipitated with trichloroacetic acid solution, containing 4 mM EDTA. The mixture was centrifuged and an aliquot of the supernatant was diluted 5000-fold with a 100 mM Tris-acetate buffer (pH 7.75)/2 mM EDTA. From this solution, 200 μl were mixed with 40 μl of ATP-monitoring reagent and bioluminescence was immediately measured in a Packard Tri-Carb 3320 liquid scintillation counter.

Lipids were extracted using the method of Rose and Oklander [15], and after removal of the solvent under a stream of nitrogen, redissolved in chloroform/methanol (1 : 1, v/v). Cholesterol was determined essentially according to Ott et al. [16]. Phospholipids were determined as inorganic phosphorus, using the method of Rouser et al. [17]. Radioactivity was measured using emulsifier scintillator solution Lipotron 56920-05010 from Kontron Analytical (Switzerland), with a Kontron Betamic II liquid scintillation counter.

The extent of replacement of erythrocyte phosphatidylcholine by DMPC was calculated based on the theoretical approach of Shipley and Clark [18] as described by Van Meer and Op den Kamp [19].

Preparation of sonicated vesicles and transfer protein. Sonicated vesicles were prepared from either phosphatidylcholine or from equimolar mixtures of phosphatidylcholine and cholesterol, essentially as described by Kuypers et al. [20]. To

prevent the sonicated vesicles from sticking to the erythrocytes, 6 mol% of egg phosphatidic acid was added to all lipid mixtures [21]. The presence of phosphatidic acid in sonicated vesicles did not affect the vesicle release from red blood cell membranes. In part of the experiments, trace amounts of [^{14}C]phosphatidylcholine and glycerol tri[^{3}H] oleate (0.1 mol% of total vesicle phosphatidylcholine) were added. Lipids were dried from chloroform/methanol mixtures (1 : 1, v/v) and subsequently dispersed in a 10 mM Tris buffer (pH 7.4), containing 144 mM NaCl, 11.1 mM glucose, 0.54 mM adenine, 12.7 mM inosine, 1 mM EDTA, penicillin ($2.0 \cdot 10^5$ IU/l) and streptomycin ($1.5 \cdot 10^5$ IU/l), referred to as incubation buffer throughout. Glucose, adenine and inosine were added to prevent ATP-depletion of erythrocytes during the incubation. The dispersions were subsequently sonicated under nitrogen using a Branson Sonifier, at 40 W, 50% duty-cycle, until opalescent (usually approx. 10–30 min). The longer periods were used for cholesterol-containing lipid mixtures. The lipid suspensions were subsequently centrifuged at $100\,000 \times g$ for 45 min in a MSE-65 Prepspin ultracentrifuge at 25°C, the supernatants being used in the incubations of red blood cells. Sonicated vesicles used for cholesterol depletion of human erythrocytes were composed of egg phosphatidylcholine and 6 mol% of egg phosphatidic acid. The phosphatidylcholine-specific phospholipid-transfer protein was purified from bovine liver [22] and pretreated as described before [20]. The specific activity was 1.2 μmol phosphatidylcholine exchanged/mg per min.

Incubation procedures. All incubations were carried out at 30°C under gentle agitation at a hematocrit of 25–30%. DMPC-induced vesiculation was carried out essentially as previously described [5]. Incubations with phosphatidylcholine-specific phospholipid-transfer protein were performed according to Kuypers et al. [20]; final protein concentration 2–3 μM. In all incubations, the molar ratio of sonicated vesicle phosphatidylcholine to erythrocyte phosphatidylcholine was between 2 : 1 and 8 : 1. Replacement of sonicated lipid vesicles in an incubation mixture was performed as follows. The reaction mixture was centrifuged (Sorvall RT-6000, 3000 rpm, 15 min, 25°C) and the supernatant was carefully removed. The pellet was washed twice in incubation buffer to remove contaminating sonicated vesicles, and subsequently resuspended in the same amount of buffer, containing the new population of sonicated vesicles for a subsequent incubation. During the incubation duplicate samples were taken at 30-min intervals. One sample was used to determine the total acetylcholinesterase activity in the incubation mixture as well as for lipid analyses. The second sample was centrifuged at $6000 \times g$ in a Hettich Mikroliter Centrifuge for 3 min at ambient temperature to pellet the red blood cells. Acetylcholinesterase, an integral protein of the red cell membrane that has been shown to be associated with the released vesicles [5,6,23], was used as a membrane marker and its activity found in the supernatant was taken as a measure for the release of membrane vesicles as previously described [1,5]. Pelleted erythrocytes were washed in a 5-fold excess of incubation buffer and, after resuspension, used for lipid analyses.

Cholesterol depletion of erythrocyte membranes was performed by incubation of erythrocytes with sonicated egg phosphatidylcholine vesicles for 3–4 h as described by Giraud et al. [13].

Results

In agreement with earlier findings [5] release of membrane vesicles from human erythrocytes was obtained upon incubation of the cells with sonicated DMPC vesicles (Fig. 1). The release of membrane vesicles, determined as vesicle-bound acetylcholinesterase, started after a lag period of 60–90 min, and after about 6 h incubation a plateau was reached when approx. 75% of the total acetylcholinesterase from the erythrocyte membrane was found in the supernatant. During this incubation period, the red blood cell membrane cholesterol level decreased to approx. 80% of its original value. On the other hand, when erythrocytes were incubated with sonicated vesicles composed of equimolar amounts of DMPC and cholesterol, no significant release of membrane vesicles took place (approx. 1% during a 6-h incubation; Fig 1) and no decrease in red blood cell membrane cholesterol levels was observed. The same result was obtained when erythrocytes were incubated with sonicated egg phosphati-

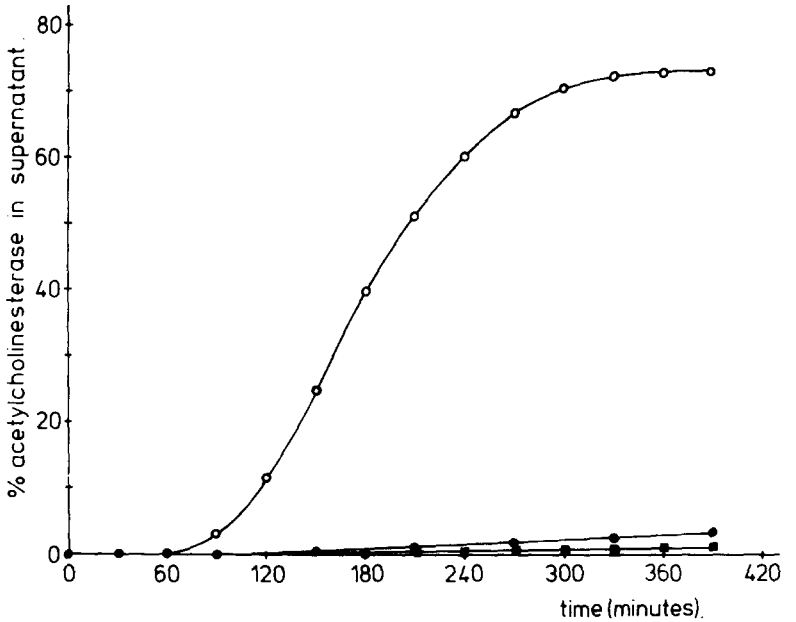

Fig. 1. Incubation of human erythrocytes with sonicated vesicles of various lipid composition in absence of phospholipid-transfer protein. Sonicated lipid vesicles had the following composition: ○, DMPC; ●, DMPC/cholesterol (1 : 1, mol/mol); □, egg phosphatidylcholine; ■, egg phosphatidylcholine/cholesterol (1 : 1, mol/mol), both superimposed. Incubations were carried out as described in Materials and Methods.

dylcholine/cholesterol vesicles (molar ratio of phosphatidylcholine to cholesterol, 1 : 1, Fig. 1). Incubation of red blood cells with pure egg phosphatidylcholine vesicles decreased membrane cholesterol levels to approx. 85% within 3 h but did not cause a measurable vesicle release (Fig. 1).

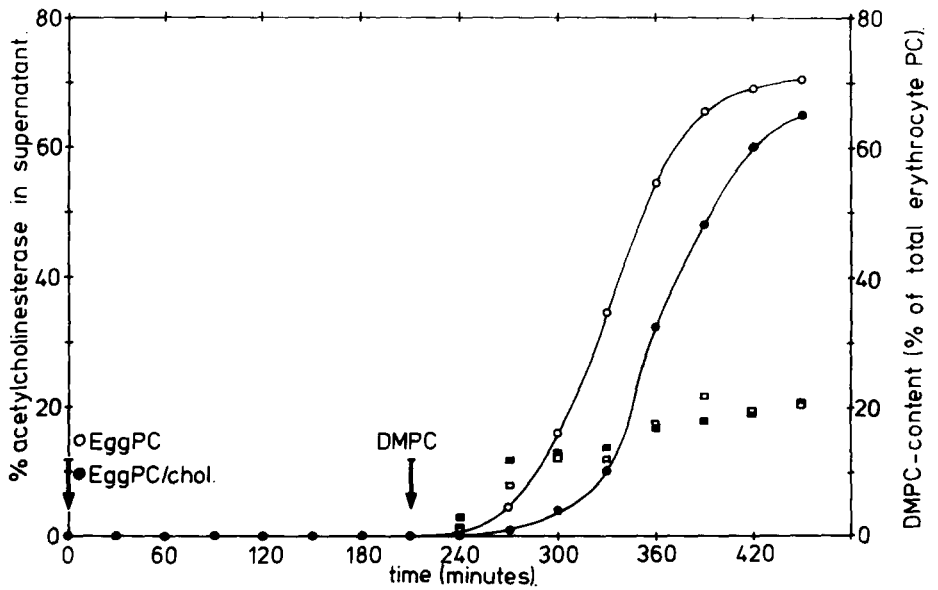

Fig. 2. Incubation of human erythrocytes with sonicated DMPC vesicles in absence of phospholipid-transfer protein after previous modulation of membrane cholesterol levels. During the first 3 h erythrocytes were incubated with sonicated lipid vesicles composed of either egg phosphatidylcholine (○), or egg phosphatidylcholine/cholesterol (●) (1 : 1, mol/mol). After removal of these vesicles, cells were subsequently incubated with sonicated vesicles composed of pure DMPC. The DMPC content of erythrocyte membranes is expressed as percent of total red cell phosphatidylcholine (□, ■). Open symbols represent the incubation with egg phosphatidylcholine vesicles, followed by DMPC vesicles, and filled symbols represent the incubation with egg phosphatidylcholine/cholesterol vesicles, followed by DMPC vesicles. Incubations were carried out as described in Materials and Methods.

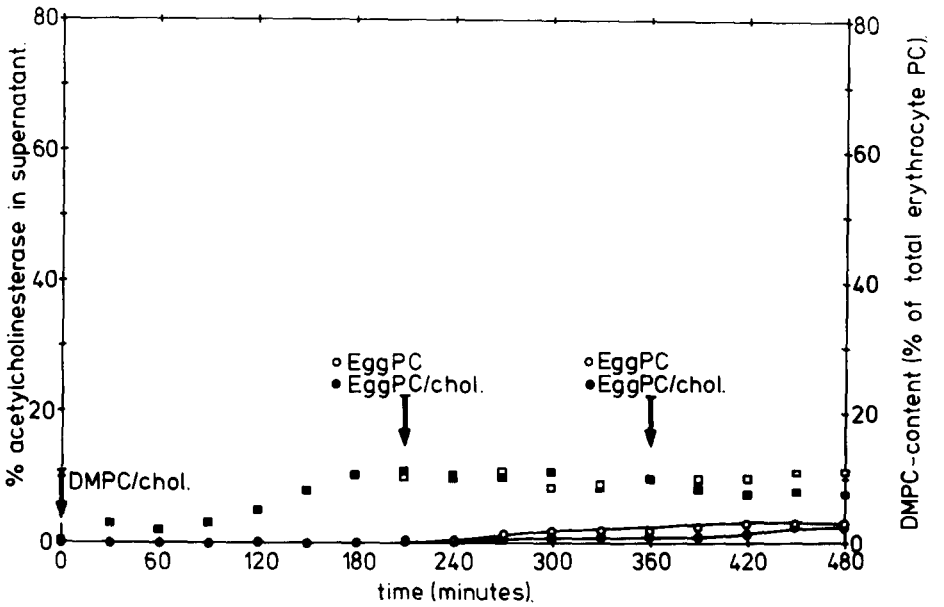

Fig. 3. Incubation of human erythrocytes with sonicated DMPC/cholesterol vesicles in absence of phospholipid-transfer protein followed by cholesterol depletion. During the first 3 h erythrocytes were incubated with sonicated DMPC/cholesterol vesicles (1 : 1, mol/mol). After removal of those vesicles, cells were subsequently incubated with sonicated vesicles composed of either egg phosphatidylcholine (○), or egg phosphatidylcholine/cholesterol (●) (1 : 1, mol/mol); these vesicles being replaced by new batches after another 2 h of incubation. The DMPC content of erythrocyte membranes is expressed as percent of total red cell phosphatidylcholine (□, ■). Open symbols represent the incubation with DMPC/cholesterol vesicles, followed by egg phosphatidylcholine vesicles, and filled symbols represent the incubation with DMPC/cholesterol vesicles, followed by egg phosphatidylcholine/cholesterol vesicles. Incubations were carried out as described in Materials and Methods.

In a two-step incubation procedure, the cholesterol level of the erythrocytes was first decreased to about 85% by incubation of erythrocytes with sonicated egg phosphatidylcholine vesicles for 3 h. Subsequent incubation with sonicated DMPC vesicles resulted in a significantly shortened lag period before vesiculation started (Fig. 2). When the preincubation was carried out with sonicated vesicles composed of equimolar amounts of cholesterol and egg phosphatidylcholine, not leading to a reduction in cholesterol content of the red cell membrane, the normal lag period upon the addition of sonicated DMPC vesicles was observed (Fig. 2). In both experiments, acetylcholinesterase recovered in the supernatant again reached a final plateau of about 75% (Fig. 2). The extent of replacement of erythrocyte phosphatidylcholine by DMPC was not influenced by these pretreatments of the cells and was approx. 18% after a 3-h incubation in either case (Fig. 2).

Preincubation of the cells with sonicated DMPC/cholesterol (1 : 1, mol/mol) vesicles, followed by two subsequent incubations with sonicated cholesterol-free egg phosphatidylcholine vesicles, did not give rise to an appreciable release of membrane vesicles (Fig. 3). During the 3-h incubation with DMPC/cholesterol vesicles, only about 10% of the native red cell phosphatidylcholine had been replaced by DMPC (Fig. 3).

From this result, it can be concluded that the transfer of DMPC from sonicated phospholipid/cholesterol vesicles proceeds less readily than from cholesterol-free DMPC vesicles. Hence, the further incubations were carried out in presence of a phosphatidylcholine-specific phospholipid-transfer protein, to ensure enhanced and reproducible phospholipid-transfer rates. Neither the onset of vesiculation nor the plateau value that is finally reached in the release of membrane vesicles from the red cells induced by sonicated cholesterol-free DMPC, is affected by the presence of the transfer protein (Fig. 4). However, the release process itself proceeded significantly faster in the presence of the transfer protein. On the other hand, incubation

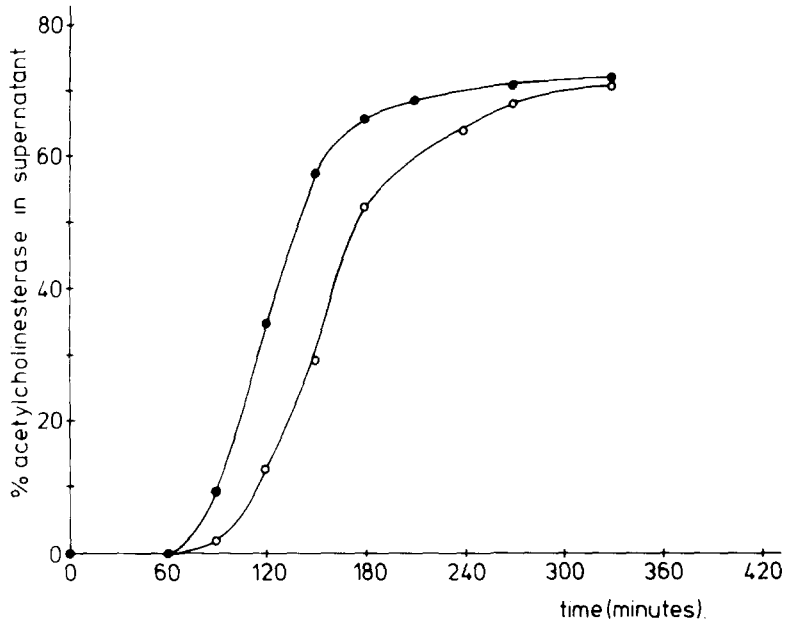

Fig. 4. DMPC-induced release of membrane vesicles from human erythrocytes in the presence or absence of the phosphatidylcholine-specific phospholipid-transfer protein. Incubations were carried out as described in Materials and Methods. Both curves were obtained with erythrocytes from the same donor and incubated in two parallel experiments either in presence (●) or absence (○) of the phosphatidylcholine-specific phospholipid-transfer protein.

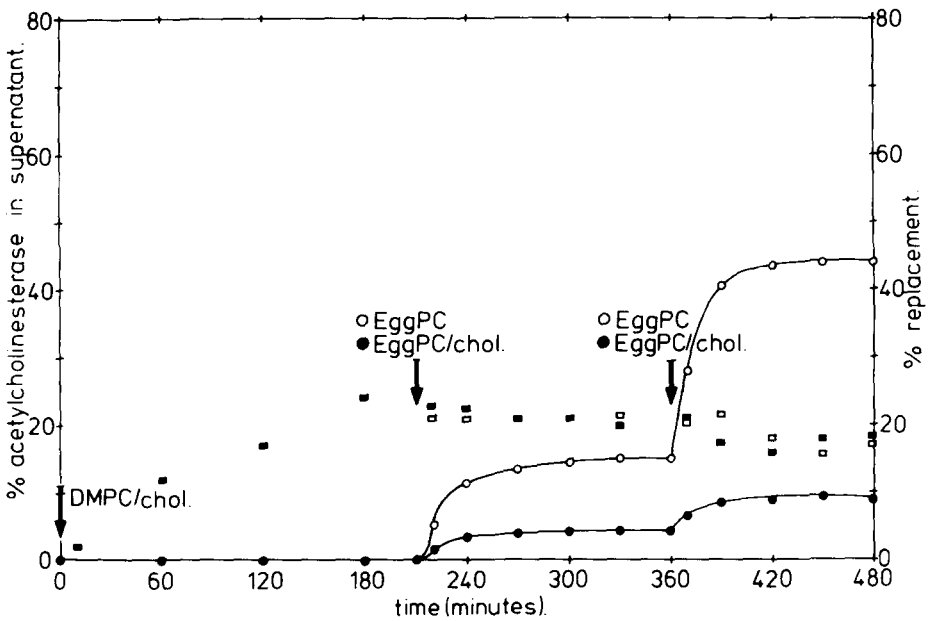

Fig. 5. Incubation of human erythrocytes with sonicated DMPC/cholesterol vesicles in presence of phospholipid-transfer protein, followed by cholesterol depletion. During the first 3 h, erythrocytes were incubated with DMPC/cholesterol vesicles (1 : 1, mol/mol) in the presence of the phosphatidylcholine-specific transfer protein. After removal of those vesicles, cells were subsequently incubated with sonicated vesicles composed of either egg phosphatidylcholine (○), or egg phosphatidylcholine/cholesterol (●) (1 : 1, mol/mol); these vesicles being replaced by new batches after another 2 h of incubation. The extent of replacement of erythrocyte phosphatidylcholine by DMPC is expressed as percent of total red cell phosphatidylcholine (□, ■). Open symbols represent the incubation with DMPC/cholesterol vesicles, followed by egg phosphatidylcholine vesicles, and filled symbols represent the incubation with DMPC/cholesterol vesicles, followed by egg phosphatidylcholine/cholesterol vesicles. Incubations were carried out as described in Materials and Methods.

with sonicated DMPC/cholesterol vesicles (1 : 1, mol/mol), in the presence of the transfer protein did not result in any significant membrane vesicle release (Fig. 5), not even when incubations were carried out for up to 5 h (results not shown). The extent of replacement of red cell phosphatidylcholine by DMPC was slightly over 20% (Fig. 5). When these DMPC-loaded cells were subsequently incubated with sonicated egg phosphatidylcholine vesicles, to reduce the cholesterol content in their membranes, an instantaneous release of vesicles was observed (Fig. 5). The acetylcholinesterase activity in the supernatant reached a value of approx. 18% during treatment with a first batch of sonicated egg phosphatidylcholine vesicles and was increased to more than 40% when this batch had been replaced by a second one. With sonicated egg phosphatidylcholine/ cholesterol vesicles (1 : 1, mol/mol), which do not alter red cell membrane cholesterol levels, the extent of vesiculation was greatly decreased, as no more than 10% of the acetylcholinesterase activity was found in the supernatant.

Discussion

Erythrocyte membrane vesiculation is known to occur under various in vitro conditions [1,2,5,24]. It appears that at least two independent processes are required to reach the stage of vesiculation. In all these processes, the first step observed is the formation of echinocytes. However, such echinocytosis is also observed as a consequence of treatments with other membrane-penetrating agents that do not induce vesicle release. Hence, the question arises what makes a cell progress to a point where it sheds vesicles. Up to now, this second process, initiating membrane vesiculation of previously formed echinocytes, is not yet fully understood. It is well-known that incubation of red blood cells with sonicated phosphatidylcholine vesicles decreases the cholesterol level in their membranes [13]. Therefore, the possibility has to be considered that, besides formation of echinocytes, cholesterol depletion is an important prerequisite of DMPC-induced vesiculation. This notion arises from the observation that vesicle release is suppressed in all incubations where the cholesterol level of the membrane is not altered (Figs. 1, 3 and 5), although the uptake of DMPC into the membrane is clearly demonstrated by use of radiolabelled DMPC (Figs. 3 and 5). Under these conditions, only crenation of the erythrocyte will take place [12]. On the other hand, erythrocytes containing appreciable amounts of DMPC will release vesicles as soon as their cholesterol level is decreased (Fig. 5). Similarly, the lag period observed between the addition of DMPC and the onset of vesiculation is clearly reduced when membrane cholesterol levels are previously decreased to approx. 85% of the normal value before the exogenous phospholipid is added (Fig. 2). These results emphasize the importance of membrane cholesterol levels in the vesiculation process and show that crenation itself, not being accompanied by cholesterol depletion, is not sufficient to initiate membrane vesiculation. Cholesterol depletion achieved by incubation of erythrocytes with sonicated phosphatidylcholine vesicles slows down as the cholesterol content of the acceptor vesicles increases in time. This explains the plateau value observed in vesicle release upon incubation of DMPC-enriched erythrocytes with sonicated cholesterol-free egg phosphatidylcholine vesicles (Fig. 5). Obviously replacement of these acceptor vesicles by a fresh dispersion of sonicated egg phosphatidylcholine causes a further decrease in cholesterol content of the red cells, concomitant with a further vesiculation of their membranes.

It is most obvious that release of membrane vesicles from the intact red cell involves a fusion process between two halves of the inner monolayer, approaching each other very closely. This may be established during echinocytosis of the cell, which is induced in our studies by the incorporation of DMPC into the membrane. The fact that the vesicles ultimately released from the cell have been shown to be essentially free of skeletal proteins [23], strongly suggests that this protein network is already absent at those places where the fusion process is going to take place. Of course, this is another prerequisite to establish an intimate contact between the two halves of the inner lipid leaflet. Another and equally important prerequisite that should be fulfilled is a destabilization of the lipid bilayer to trigger the fusion process. Such processes have been reported to be critically dependent on an appropriate cholesterol/phos-

pholipid ratio [25]. In the present studies, this ratio is apparently established by a decrease in the cholesterol content of the membrane. In a recent report, Chabanel et al. [26] suggested that a decrease in red cell membrane cholesterol primarily increases the fluidity of the inner leaflet of the membrane that is highly enriched in the aminophospholipids, phosphatidylserine and phosphatidylethanolamine [27]. Phosphatidylethanolamine is known to readily adopt a non-bilayer configuration, such as the hexagonal H-II phase, unless it is sufficiently stabilized in a bilayer configuration by other components. Hence, it is tempting to speculate that a moderate cholesterol depletion of the erythrocyte results in an increased tendency of the red cell membrane phosphatidylethanolamine to adopt a non-bilayer configuration. Indeed, such configurations have been considered as an intermediate step in membrane fusion processes [28,29].

So far, phosphatidylcholine-induced vesiculation of erythrocytes has only been demonstrated upon incubation of the cells with sonicated DMPC or dilauroylphosphatidylcholine vesicles, but does not take place when dipalmitoylphosphatidylcholine is used (Frenkel, E.J., et al., unpublished observation) although the latter lipid also induces the formation of echinocytes [12]. Furthermore, also the amount of DMPC incorporated into the red cell membrane appears to be critical, since only limited vesiculation is observed when no more than 10% of the red cell membrane phosphatidylcholine has been replaced by DMPC (Fig. 3).

In conclusion, the present study shows that formation of echinocytes as well as a reduction of the red cell membrane cholesterol level are necessary prerequisites for DMPC-induced vesiculation of the cells. Further studies are now under way to characterize the structural properties of crenating agents that are required to induce vesicle release in combination with membrane cholesterol depletion, and to define the extent of cholesterol depletion that is needed to obtain an optimal cholesterol/phospholipid ratio for vesiculation and hence, membrane fusion to occur.

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