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Modulation of erythrocyte vesiculation by amphiphilic drugs

Peter Bütikofer, Urs Brodbeck and Peter Ott

Institut für Biochemie und Molekularbiologie der Universität Bern, Bern (Switzerland)

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Release of acetylcholinesterase-containing vesicles from human erythrocyte membranes induced by dimyristoylphosphatidylcholine (DMPC) was inhibited by exposure of red cells to cationic amphiphilic drugs like tetracaine, chlorpromazine and primaquine which all are known to induce stomatocyte formation. On the other hand, the process was facilitated when red cells were exposed to crenators like the anionic drugs indomethacine and phenylbutazone or when DMPC was added to calcium-loaded red cells. The results suggest that agents which are known to modulate red cell shape do also influence the vesiculation behavior of the cells.

Introduction

Release of membrane vesicles from human erythrocytes has been described to occur under various in vivo and in vitro conditions [1–5]. All procedures to induce red cell vesiculation have in common that in an initial step erythrocytes change their morphology and become spiculated cells. Therefore, echinocytic shape transformation has to be considered as a prerequisite for vesicle release.

Red cell shape changes can be induced by various amphiphilic drugs that intercalate with the erythrocyte membrane [6–9]. The effect of an individual drug on erythrocyte morphology can largely be predicted by the bilayer couple hypothesis proposed by Sheetz and Singer [6]. Generally, drugs with an anionic head group (so called crenators) induce shape change of red cells from discocytes to echinocytes (nomenclature according to Bessis [10]), whereas drugs with a cationic head group (so called cup formers) induce transformation to stomatocytes [6–9, 11]. Crenators and cup formers may act as antagonists, e.g. crenated red cells can (partially) be converted back to discocytes by exposure to a cup forming drug [6–9,11].

Since vesicle release is preceded by a red cell shape change one would expect that agents which are known to affect this shape change would also affect the vesiculation process. In order to test this hypothesis, we investigated the effects of various amphiphilic drugs on dimyristoylphosphatidylcholine (DMPC)-induced vesiculation of human erythrocytes, which is a relatively well characterized method to induce vesicle release from red cell membranes [3,12,13].

Materials and methods

Erythrocytes. Fresh human blood samples from healthy adult donors were obtained from the

Abbreviation: DMPC, dimyristoylphosphatidylcholine.

Correspondence: P. Ott, Institut für Biochemie und Molekularbiologie der Universität Bern, Bühlstrasse 28, CH-3012 Bern, Switzerland.

Central Blood Bank of the Swiss Red Cross. Erythrocytes were separated from plasma by centrifugation at 2500 rpm for 10 min at 4°C and washed three times with 10 mM Tris-HCl (pH 7.4) containing 144 mM NaCl. Each time the buffy coat was carefully removed. Red cells were immediately used for vesiculation experiments.

Reagents. Dimyristoylphosphatidylcholine (DMPC), tetracaine, chlorpromazine, primaquine, indomethacine and phenylbutazone were obtained from Sigma (St. Louis, MO, U.S.A.). 1,2-Di[1-^{14}C]myristoylphosphatidylcholine and glycerol tri[9,10(n)-^{3}H]oleate were supplied by Amersham International (Amersham, U.K.). Emulsifier scintillator solution Lipotron was from Kontron Analytical (Zürich, Switzerland). Penicillin was purchased from Novo Industri (Copenhagen, Denmark) and streptomycin-sulfate was from Grogg Pharmaceutical products (Bern, Switzerland). All other reagents were standard commercial products obtained either form Fluka AG (Buchs, Switzerland) or from Merck (Darmstadt, F.R.G.) and were of the highest purity available.

Assays. Acetylcholinesterase activity was determined according to Ellman et al. [14]. Lipids were extracted using the method of Rose and Oklander [15] and phospholipid phosphorus was determined according to Rouser et al. [16]. Radioactivity was determined with a Kontron Betamatic II liquid scintillation counter.

Erythrocyte vesiculation. DMPC-induced release of acetylcholinesterase-containing vesicles was carried out as described before [3,12,13]. Briefly, washed erythrocytes (8% final hematocrit) were incubated in presence of sonicated suspensions of DMPC (0.5 mg/ml final concentration) in 10 mM Tris-HCl, supplemented with 144 mM NaCl, and with glucose, adenine, inosine and EDTA [13] (referred to as incubation buffer) at 30°C. At appropriate time intervals aliquots were removed and red cells were pelleted. Vesicle release was monitored by measuring acetylcholinesterase activity in the cell free supernatant. Amphiphilic drugs were added to red cells from stock solutions in methanol (tetracaine, 500 mM; indomethacine, 25 mM) or incubation buffer (chlorpromazine, 100 mM; primaquine, 100 mM) or were added directly to incubation buffer (phenylbutazone). In order to determine the rate of incorporation of DMPC into erythrocyte membranes, trace amounts of [^{14}C] DMPC and glycerol tri[^{3}H]oleate were added to the lipid dispersion before sonication, exactly as described elsewhere [12].

Treatment of red cells with calcium and ionophore A23187 (Ca^{2+}-loading) was performed essentially according to Allan et al. [1]. Briefly, washed erythrocytes (16% final hematocrit) were incubated in incubation buffer without EDTA, supplemented with 0.8 mM Ca^{2+} (final concentration) and 4 μM ionophore (final concentration) at 37°C. At appropriate times, aliquots were removed and added to small volumes of 100 mM EDTA in incubation buffer in order to stop vesiculation [17]. Red cells were subsequently pelleted, and acetylcholinesterase activity was measured in the supernatant as described above. When combined treatments with DMPC were used, Ca^{2+}-loaded erythrocytes were treated with EDTA (see above) and subsequently added to an equal volume of DMPC (0.5 mg/ml final concentration). Incubation was continued at 30°C and vesicle release was monitored by measuring acetylcholinesterase in the cell free supernatant.

Hemolysis during vesiculation experiments never exceeded 2% at the end of incubation.

The morphology of erythrocytes (fixed in 2% glutaraldehyde) was monitored by light microscopy using Zeiss Nomarsky optics.

Results

Human erythrocytes incubated with sonicated suspensions of DMPC have been shown to undergo shape change from discocytes to echinocytes and subsequently release membrane vesicles [3]. This process is terminated after 6 h of incubation at 30°C when 60–80% of acetylcholinesterase (which is used as a marker to follow erythrocyte vesiculation) are found in the cell free supernatant (Fig. 1; see also Refs. 3, 12 and 13). On the other hand, in presence of the cationic drugs tetracaine (0.5–2.0 mM), chlorpromazine (0.1–0.2 mM), or primaquine (0.5–2.0 mM) – which are known as cup formers – the extent of acetylcholinesterase release was reduced by 20% to 100% as compared to the release measured in absence of drug (Fig. 1). A similar effect was observed when the drugs were added to red cells only shortly before

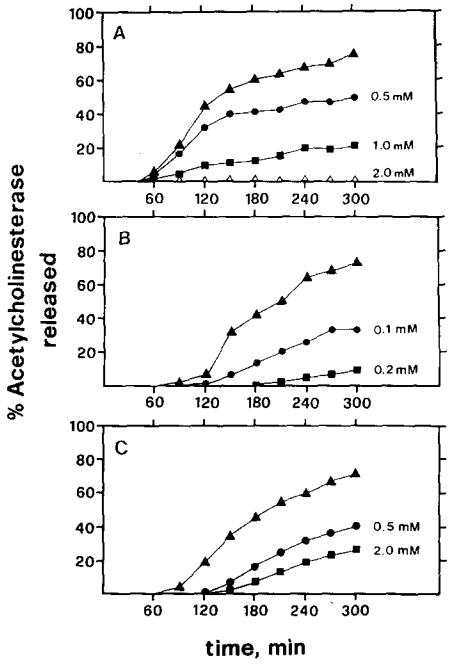

Fig. 1. DMPC-induced vesiculation of human erythrocytes in presence of cationic drugs. Red cells were incubated with sonicated suspensions of DMPC and release of membrane vesicles was followed by measuring acetylcholinesterase in the cell free supernatant. For details see Materials and Methods. Acetylcholinesterase activity in the vesicle fraction was expressed as % of total activity in the suspension. The total enzyme activity remained constant during each experiment. (A) tetracaine, (B) chlorpromazine and (C) primaquine were added at the begin of incubation in the concentrations indicated. ▲, represents control erythrocytes that were incubated with DMPC in the absence of drugs.

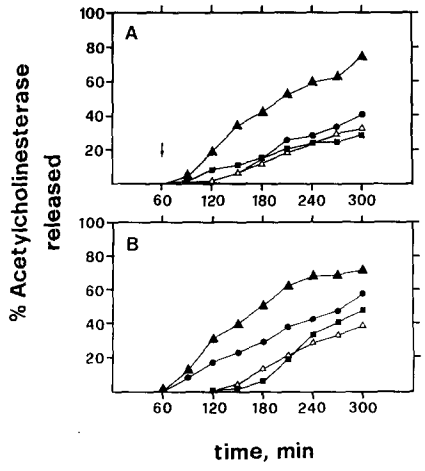

Fig. 2. Effect of cationic drugs on DMPC-induced vesiculation of human erythrocytes. Experimental conditions were as described in Fig. 1. (A) ■, Tetracaine (1 mM); △, chlorpromazine (0.2 mM); and (●) primaquine (2 mM), respectively, (final concentrations are indicated) were added to red cells after 60 min of incubation in presence of DMPC. ▲, represents control erythrocytes that were incubated with DMPC in the absence of drugs. (B) Red cells were preincubated with: ■, tetracaine (4 mM); △, chlorpromazine (0.2 mM); and ●, primaquine (4 mM), respectively, for 30 min at 30 °C in incubation buffer without DMPC. ▲, represents control erythrocytes that were preincubated in the absence of drugs. The cells were then washed in 2×10 volumes of cold incubation buffer, and subsequently DMPC was added.

DMPC-induced vesicle release started (Fig. 2A). The process was also inhibited when red cells were preincubated with the drugs and the subsequent incubation with DMPC was carried out in a drug-free incubation buffer (Fig. 2B). However, as compared to the incubations performed throughout in presence of the drugs, 2–8-fold higher concentrations had to be used to obtain a significant decrease of vesicle release.

On the other hand, incubation of red blood cells with DMPC in presence of the anionic drugs indomethacine (0.2–0.5 mM) and phenylbutazone (1.0–2.0 mM) –which are known as crenators– resulted in a shortened lag time between the addition of DMPC and the onset of vesiculation as

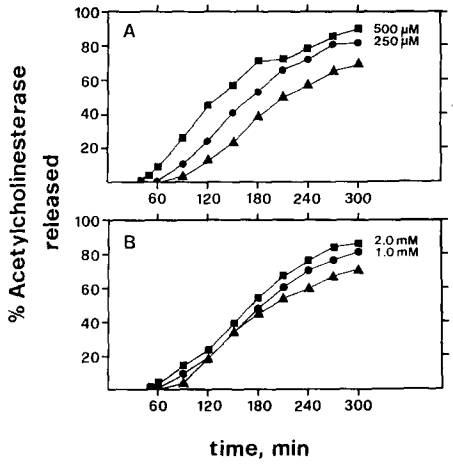

Fig. 3. DMPC-induced vesiculation of human erythrocytes in presence of anionic drugs. Experimental conditions were as described in Fig. 1. (A) Indomethacine and (B) phenylbutazone, respectively, were added at the begin of incubation in the concentrations indicated. ▲, represents control erythrocytes that were incubated with DMPC in the absence of drugs.

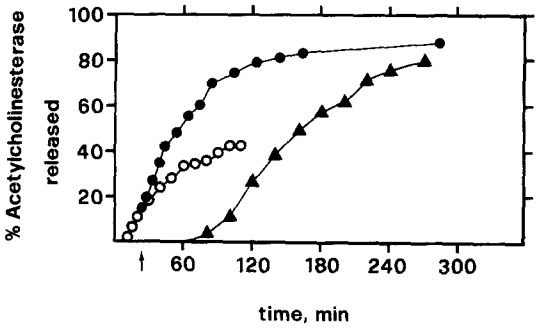

Fig. 4. DMPC-induced vesiculation of calcium loaded human erythrocytes. Calcium loading of red cells was carried out as described in Materials and Methods. After 3 min (▲) and 24 min (●), respectively, aliquots of calcium loaded erythrocytes were removed and calcium induced vesiculation was blocked by adding EDTA. Subsequently, DMPC was added and vesiculation was monitored as described in Fig. 1. (○) represent vesicle release from calcium loaded red cells in absence of DMPC.

well as in a moderate increase in the extent of acetylcholinesterase release (Fig. 3). a significant stimulation of the process was observed when DMPC was added to echinocytic red cells that were obtained by preincubation in presence of calcium and ionophore A23187 for 24 min (Fig. 4). No stimulation of DMPC-induced vesiculation was observed (Fig. 4) when this preincubation was carried out for only 3 min, which did not result in formation of echinocytes (result not shown).

In all experiments the total activity of the marker enzyme acetylcholinesterase in the incubation mixtures was not affected by amphiphilic drugs, calcium or EDTA.

Discussion

According to the bilayer couple hypothesis [6], shape transformations of human erythrocytes after exposure to amphiphilic drugs can largely be predicted by the nature of the added molecules. Cationic drugs accumulate at the inner half of the red cell membrane and induce membrane invagination, whereas anionic drugs are preferentially incorporated in the outer half and induce membrane externalization [6].

It has to be assumed that factors which influence red cell shape should also have an effect on the vesiculation process since DMPC-induced vesiculation of red blood cells is preceded by formation of echinocytes [3]. The present report confirms this hypothesis and demonstrates that a series of amphiphilic drugs are able to modulate DMPC-induced release of acetylcholinesterase-containing vesicles from human erythrocytes. Cup formers obviously counteract the tendency of DMPC to form echinocytes [18] and therefore inhibit vesicle release (Fig. 2). A similar situation has been observed with heat-treated red cells where vesicle release was inhibited by tetracaine [19]. On the other hand, crenators facilitate vesiculation (Fig. 3), which is also in line with the observation that DMPC-induced vesiculation appears significantly facilitated in red cells that have been crenated by the action of calcium (Fig. 4).

Besides the effect of amphiphilic drugs on red cell shape a direct influence on the erythrocyte lipid bilayer has recently been demonstrated. Such drugs have been reported to alter the transbilayer distribution and mobility of red cell membrane phospholipids [20,21]. In DMPC-induced vesiculation, part of the exogenous lipid is incorporated into the erythrocyte membrane [12,13]. Due to the slow transbilayer mobility of phosphatidylcholine [22], it must be assumed that the exogenous lipid will accumulate in the outer half of the lipid bilayer, thereby inducing a shape transformation of erythrocytes to spiculated cells [13]. Since amphiphilic drugs are able to increase the transbilayer mobility of erythrocyte membrane phospholipids [20,21], this would also result in an accelerated random distribution of DMPC between the two membrane leaflets. Hence, formation of echinocytes and release of membrane vesicles would be inhibited.

Such a mechanism also helps to explain why crenators show a relatively limited effect on the vesiculation process. On one hand their tendency to promote crenation supports vesiculation and on the other hand their possible influence on the membrane lipid bilayer would promote a random distribution of DMPC. Both effects together may well result in a moderate stimulation of vesicle release (Fig. 3).

It has to be considered that cationic drugs interact directly with DMPC vesicles in suspension [23,24], and thus may influence vesiculation

by modification of DMPC vesicle behavior rather than through a direct intercalation with the red cell membrane. This possibility, however, can largely be ruled out since incorporation of DMPC into erythrocyte membranes –measured with radioactively labeled DMPC– was comparable in presence and absence of tetracaine (result not shown). Furthermore, cationic drugs inhibit vesiculation also when added only after 60 min of incubation (Fig. 2A) i.e. at a time when a considerable amount of DMPC has already penetrated the cells [12,13]. Finally, cationic drugs also inhibit vesicle release when red cells are only pretreated with the drug and subsequently incubated with DMPC in drug-free buffer (Fig. 2B), which again indicates direct interaction of the drugs with the erythrocyte membrane.

Taken together, the results presented here suggest that cationic drugs which induce a stomatocytic shape transformation of red cells act as inhibitors of membrane vesiculation by preventing erythrocytes to become spiculated cells, and crenators accelerate the vesiculation process by facilitating echinocytic shape transformation of red cells.

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