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ORIGINAL ARTICLE

The phospholipid composition and cholesterol content of platelet-derived microparticles: a comparison with platelet membrane fractions

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Summary. *Background:* The processes that govern the distribution of molecules between platelets and the microparticles (MP) they release are unknown. Certain proteins are sorted selectively into MP, but lipid sorting has not been studied. *Objectives:* To compare the phospholipid composition and cholesterol content of platelet-derived MP obtained with various stimuli with that of isolated platelet membrane fractions. *Methods:* Washed platelets from venous blood of healthy individuals ($n = 6$) were stimulated with collagen, thrombin, collagen plus thrombin, or A23187. Platelet activation, MP release and antigen exposure were assessed by flow cytometry. MPs were isolated by differential centrifugation. Platelet plasma-, granule- and intracellular membranes were isolated from platelet concentrates ($n = 3$; 10 donors each) by pressure homogenization and Percoll density gradient fractionation. The phospholipid composition and cholesterol content of MPs and membrane fractions were analyzed by high performance thin layer chromatography. *Results:* The phospholipid composition of MPs was intermediate compared with that of platelet plasma- and granule membranes, and differed significantly from that of intracellular membranes. There were small but significant differences in phospholipid composition between the MPs produced by the various agonists, which paralleled differences in P-selectin exposure in case of the physiological agonists collagen, thrombin, or collagen plus thrombin. The cholesterol content of MPs tended to be higher than that of the three-platelet membrane fractions. *Conclusions:* Regarding its phospholipid content, the MP membrane is a composite of the platelet plasma- and granule membranes, showing subtle differences depending on the platelet agonist. The higher cholesterol content of MPs suggests their enrichment in lipid rafts.

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Introduction

Platelet-derived microparticles (MP) are intricately involved in many physiological and pathophysiological processes. First described by Wolf in 1967 as 'platelet dust', they have been recognized from the very beginning to play a role in blood coagulation [1]. By exposing negatively charged phospholipids and binding sites for various coagulation factors [2–6], they support the assembly and optimal function of coagulant enzyme complexes. They can also expose tissue factor [7], which has been shown to be active *in vitro* [8] as well as *in vivo* [9]. Besides their role in coagulation, platelet-derived MPs transport and transfer bioactive molecules, activate other cells, and contribute to inflammatory processes in various ways [10–13].

Although the functional importance of platelet-derived MPs is clear, little is known about the mechanism of their formation and especially the processes that govern the distribution of molecules between the MP that is being released and the remaining platelet. During activation of platelets, an increase in cytoplasmic Ca^{2+} concentration is followed by a reorganization of the cytoskeleton, shape change, and exocytosis of the contents of platelet granules (α-granules, dense granules and lysosomes). Exocytosis implies fusion of granule membranes with the open canalicular system or the plasma membrane [14,15]. MPs are thought to be formed by budding of the surface membrane, in a process that also involves proteolysis of components of the membrane skeleton [16,17]. Certain platelet proteins are selectively shed into released vesicles [4–6,18], and the same phenomenon has been described for other cell types as well [19–23]. Whether membrane lipids are also selectively sorted into MPs is unknown. Here, we therefore determined the phospholipid composition and cholesterol content of platelet-derived MPs obtained with various stimuli, and compared them with isolated platelet plasma-, granule- and intracellular membranes.

Methods

Activation of platelets and isolation of platelet-derived MPs

Venous blood was obtained from six healthy individuals who had not taken any medication during the previous 10 days and had given their informed consent. The blood was collected into 1/10th volume of 105 mmol L^{-1} trisodium citrate, and centrifuged at 180 *g* for 15 min at 20 °C to obtain platelet-rich plasma (PRP). PRP was acidified with 1/6th volume of acid citrate dextrose (ACD; 71 mmol L^{-1} citric acid, 85 mmol L^{-1} trisodium citrate and 110 mmol L^{-1} D-glucose, initial pH approximately 4.4). The acidified PRP, pH approximately 6.5, from each healthy individual was divided into two aliquots. To one of the aliquots, dibutyryl cAMP (dbcAMP) was added (final concentration 2 mmol L^{-1}), to the other nothing, and both PRP fractions were incubated for 20 min at 37 °C. Platelets were then pelleted by centrifugation at 700 *g* for 20 min at 20 °C, then washed once with a buffer containing 137 mmol L^{-1} NaCl, 2.6 mmol L^{-1} KCl, 1.0 mmol L^{-1} $MgCl_2$, 11.9 mmol L^{-1} $NaHCO_3$, 5.6 mmol L^{-1} D-glucose, 1 mmol L^{-1} EDTA (buffer A; pH 6.5). For the platelet suspensions pretreated with dbcAMP, this buffer also contained 2 mmol L^{-1} dbcAMP. Finally, platelets were resuspended in buffer A without EDTA and without dbcAMP (pH 7.35).

To the platelet suspensions treated with dbcAMP, nothing was further added (inactivated platelets). Aliquots of the platelet suspensions not treated with dbcAMP were activated in the presence of 2.5 mmol L^{-1} $CaCl_2$ with (i) 20 μg mL^{-1} collagen (Chrono-Log Corp., Havertown, PA, USA), (ii) 1 U mL^{-1} thrombin (Sigma, St Louis, MO, USA), (iii) 20 μg mL^{-1} collagen plus 1 U mL^{-1} thrombin, or (iv) 2.5 μmol L^{-1} calcium ionophore A23187 (Calbiochem, San Diego, CA, USA). As a control, 2.5 mmol L^{-1} $CaCl_2$ alone was also added to aliquots of the platelet suspensions neither treated with dbcAMP, nor with agonists. The mixtures were incubated at 37 °C for 30 min, without stirring, to avoid major platelet aggregation. Activation was stopped by the addition of 2.5 mmol L^{-1} final concentration of EDTA, and aliquots were taken from each tube for flow cytometric analysis of platelets and MPs (see below). Platelet counts were determined with the hematology analyzer CELL-DYN 4000 (Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, IL, USA). The platelet-MP suspensions were centrifuged at 1000 *g* for 20 min at 20 °C to pellet the platelets, and the supernatants containing the platelet-derived MPs were collected. The MPs were concentrated by centrifugation at 19 000 *g* for 60 min at 20 °C. They were then stored at −80 °C until lipid extraction and analysis.

Flow cytometry

Of the platelet-MP suspensions, 5 μL was diluted in 50 μL of HEPES buffer (137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 1 mmol L^{-1} $MgCl_2$, 20 mmol L^{-1} HEPES, 3.3 mmol L^{-1} NaH_2PO_4, 0.1% bovine serum albumin, 5.6 mmol L^{-1} D-glucose) containing a fluorescein isothiocyanate (FITC)- and a phycoerythrin (PE)-labeled monoclonal antibody, or the respective isotype-matched control antibodies. The mixtures were incubated in the dark for 30 min at 20 °C, after which the samples were fixed by addition of 1.5 mL of 0.3% (w/v) paraformaldehyde, and analyzed on a FACSCalibur flow cytometer with CellQuest Pro 4.0.2 software [Becton, Dickinson and Company (BD) Immunocytometry Systems, San Jose, CA, USA]. Acquisition was performed for 1 min per sample, during which the flow cytometer analyzed approximately 60 μL of the suspension. Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with gain settings in the logarithmic mode. Results showed that contamination of the original platelet preparations with erythrocytes and leukocytes was very low: the number of glycophorin A (erythrocyte marker) positive events was 0.5 ± 0.3 per 100 platelet marker positive events, and the number of CD45 (leukocyte marker) positive events was 0.6 ± 0.5 per 100 platelet marker positive events in the inactivated samples. Platelets and platelet-derived MPs were identified based on their platelet marker positivity (CD61) and FSC/SSC characteristics [24]. Concentrations of MPs were calculated based upon the number of events counted per unit time, the flow rate of the flow cytometer, and the net dilution of the samples during labeling and fixation. To identify marker positive events, thresholds were set based on samples incubated with similar concentrations of isotype-matched control antibodies.

IgG_1-FITC and -PE (both clone X40) were obtained from BD Immunocytometry Systems (San Jose), CD61-FITC (clone Y2/51, IgG_1) and antiglycophorin A-PE (clone JC159, IgG_1) from Dako (Glostrup, Denmark), CD62p-PE (clone CLB-Thromb/6, IgG_1) and CD63-PE (clone CLB-gran12, IgG_1) from Immunotech (Marseilles, France), and CD45-PE (clone CLB-T200/1, 15D9, IgG_1) from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands).

Isolation of platelet membrane fractions

Platelet membrane fractions were isolated as described by Mauco *et al.* [25]. Fresh platelet concentrates, obtained from the Red Cross Blood Bank, Utrecht, The Netherlands, were prepared from the blood of five healthy donors anticoagulated with citrate phosphate dextrose (CPD; 17.0 mmol L^{-1} citric acid, 89.4 mmol L^{-1} trisodium citrate, 16.1 mmol L^{-1} NaH_2PO_4, and 128.7 mmol L^{-1} D-glucose; 70 mL in 500 mL blood), and contained about 250×10^9 L^{-1} platelets. The concentrates were used within 24 h after blood collection.

For each of three experiments, two platelet concentrates of 250 mL were pooled, 0.2 mmol L^{-1} final concentration of EGTA was added, and the platelet suspension was centrifuged at 700 *g* for 20 min at 20 °C. The pelleted platelets were washed once with 250 mL of buffer containing 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 2.0 mmol L^{-1} $MgCl_2$, 0.42 mmol L^{-1} NaH_2PO_4, 11.9 mmol L^{-1} $NaHCO_3$,

0.35% (w/v) bovine serum albumin, 5.6 mmol L^{-1} D-glucose and 0.2 mmol L^{-1} EGTA (pH 6.5), then with the same buffer in the absence of EGTA. Afterwards, platelets were resuspended in 40 mL of ice-cold lysis buffer (buffer B; 25 mmol L^{-1} Tris, 100 mmol L^{-1} KCl, 3 mmol L^{-1} $MgCl_2$, 3 mmol L^{-1} ATP, pH 7.4) containing protease inhibitors (1 mmol L^{-1} benzamidine, 1 mmol L^{-1} EGTA, 1 mmol L^{-1} phenylmethylsulfonyl fluoride, 5 mmol L^{-1} ε-aminocaproic acid), and pressurized with nitrogen to 70 atm in a pressure homogenizer (Parr Instrument Company, Moline, IL, USA) for 20 min at 0–4 °C.

The homogenate was centrifuged at 1500 *g* for 15 min at 4 °C, and the supernatant (30–35 mL) was diluted in two volumes of twice concentrated ice-cold buffer B (pH 7.4), 1.72 volumes of Percoll (Amersham Biosciences, Uppsala, Sweden) and 0.28 volumes of water. The mixture was centrifuged at 79 000 *g* for 15 min at 4 °C in a Beckman L-80 ultracentrifuge using a Beckman 60Ti-rotor (Beckman Instruments Inc., Palo Alto, CA, USA). Two bands were obtained: a low density band consisting of a mixture of intracellular and plasma membranes, and a high density band consisting of the membranes of various granules [25,26]. The two bands were isolated, and the high density band was diluted in 50 mL of ice-cold buffer containing 100 mmol L^{-1} NaCl, 50 mmol L^{-1} KCl, 5 mmol L^{-1} HEPES and 5 mmol L^{-1} Tris (buffer C; pH 7.0). The low density band was mixed with two volumes of twice concentrated ice-cold buffer B and 1.72 volumes of Percoll. The pH of the mixture was adjusted to 9.6 with NaOH. A subsequent centrifugation at 79 000 *g* for 15 min at 4 °C resulted in the separation of a low density band enriched in plasma membranes and a high density band enriched in intracellular (dense tubular system) membranes [25,26]. Both bands were isolated and diluted in 50 mL of buffer C.

All three membrane fractions were centrifuged at 200 000 *g* for 60 min at 4 °C to remove the Percoll, and the membranes were collected and diluted in 25 mL of ice-cold buffer C. Plasma membranes were sonicated for 4 × 15 s at 1 min intervals on ice using an MSE sonicator (Soniprep 150, MSE Scientific Instruments, Crawley, UK) at maximal output, amplitude 30 μm. Following another centrifugation at 200 000 *g* for 60 min at 4 °C, the membranes were resuspended in 1–2 mL ice-cold buffer C and stored at −80 °C until lipid extraction and analysis.

Extraction of phospholipids and cholesterol

Lipids were extracted according to a modified procedure of Bligh and Dyer [27,28]. To the platelet membrane fractions (amount estimated based on protein concentrations) and to the MP suspensions (amount estimated based on MP concentrations determined by flow cytometry), 3 mL of methanol:chloroform (2:1) and 600–796 μL of 0.5% acetic acid were added (final volume 3.8 mL). The samples were thoroughly mixed for 30 s. Subsequently, 1 mL of chloroform and 800 μL of 0.5% acetic acid were added, the samples were mixed for another 30 s, and centrifuged at 1560 *g* for 10 min at 20 °C. The chloroform phase was collected and the aqueous phase was washed twice with 1 mL of chloroform. The three chloroform fractions were pooled and dried under a nitrogen stream. Finally, the platelet membrane- and the MP-derived lipids were dissolved in methanol:chloroform (2:1) for separation and quantitation by high performance thin layer chromatography (HPTLC).

High performance thin layer chromatography

Thin layer chromatography (TLC) was performed as we described previously [28] on HPTLC plates (Cat. No. 1.05641, Silica gel 60, 20 × 10 cm, mean particle size 5–7 μm, layer thickness 150–200 μm; Merck, Darmstadt, Germany), which were cleaned by predevelopment with methanol:ethyl acetate (6:4) in a Camag horizontal developing chamber (Merck). Prior to application of samples, the plates were activated at 130 °C for 10 min on a Camag TLC plate heater III (Merck). Samples, dissolved in methanol:chloroform (2:1), were applied as narrow bands of 3 mm length, 8 mm from the edge of the plate, using a Camag Linomat 5 (Merck) sample applicator. For separation of phospholipids, the plate was first developed in the Camag horizontal developing chamber with dichloromethane:ethyl acetate:acetone (80:16:4) for 70 mm to separate cholesterol, free fatty acids and triglycerides from the phospholipids. This prevented overloading at the 'start' position. The plate was then dried at 40 °C for 10 min. Subsequently, phospholipids were separated by development with chloroform:ethyl acetate:acetone:isopropanol:ethanol:methanol:water:acetic acid (30:6:6:6:16:28:6:2) for 55 mm. The plate was then dried at 130 °C for 5 min. For separation of cholesterol, the plate was developed in the Camag horizontal developing chamber with chloroform:ethyl acetate (3:7) for 70 mm, and was then dried at 130 °C for 5 min.

After the plates had cooled down, 10 mL of charring reagent [a mixture of 7.5% Cu-acetate (w/v), 2.5% $CuSO_4$ (w/v), and 8.5% H_3PO_4 (v/v) in water] was applied. The plates were incubated with the charring reagent for approximately 60 s under gentle rocking. Finally, the excess of charring reagent was removed by decanting, and the back of the HPTLC plates was cleaned with a tissue. The plates were then dried on the plate heater at 60 °C for 15 min. Subsequently, the temperature was increased stepwise up to 160 °C (steps of 10 °C every 2 min except for a heating time of 5 min at 80 °C), then left at 160 °C for 15 min to complete the charring. The density of the spots was analyzed by photodensitometric scanning (GS-800 Calibrated Densitometer, Bio-Rad, Hercules, CA, USA), and quantified using Quantity One software version 4.2.2 (Bio-Rad).

Phospholipid and cholesterol standards were obtained from Larodan [Malmö, Sweden; L-α-lysophosphatidylcholine (L-PC; 38–0104), sphingomyelin (SM; 56-1080), L-α-phosphatidylcholine (PC; 37–0106), L-α-phosphatidylserine (PS; 37-0160), L-α-phosphatidylinositol (PI; 37-0134), L-α-phosphatidylethanolamine (PE; 37-0126)], Sigma [L-α-lysophosphatidylethanolamine (L-PE; L4754), cholesterol (C8667)] and from Avanti Polar Lipids Inc. [Alabaster, AL, USA; L-α-lysophosphatidylserine (L-PS; 850092P)]. Chloroform, ethyl acetate,

acetone, methanol, ethanol, dichloromethane, isopropanol and acetic acid (all HPLC grade) were obtained from Merck. All other chemicals were of analytical quality.

Statistical analysis

Data were analyzed with GraphPad PRISM 3.02 (GraphPad Software, Inc., San Diego, CA, USA). Differences between groups were analyzed with one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. Correlations were determined using Pearson's correlation test. Differences and correlations were considered significant at $P < 0.05$. Data are presented as mean ± SD.

Results

Activation of platelets and isolation of platelet-derived MPs

Washed platelets from six healthy donors were incubated without stirring for 30 min at 37 °C (i) without a stimulus, after pretreatment with the platelet inhibitor dbcAMP, (ii) with 2.5 mmol L^{-1} calcium alone, or in combination with (iii) 20 μg mL^{-1} collagen, (iv) 1 U mL^{-1} thrombin, (v) 20 μg mL^{-1} collagen plus 1 U mL^{-1} thrombin, or (vi) 2.5 μmol L^{-1} calcium ionophore A23187. Platelet counts in the dbcAMP-treated suspensions were $207 \pm 46 \times 10^9$ L^{-1}, in the suspensions incubated with calcium alone $169 \pm 43 \times 10^9$ L^{-1} ($P > 0.05$, when compared with platelets treated with the inhibitor), with collagen $91 \pm 21 \times 10^9$ L^{-1} ($P < 0.001$), with thrombin $60 \pm 21 \times 10^9$ L^{-1} ($P < 0.001$), with collagen and thrombin $134 \pm 33 \times 10^9$ L^{-1} ($P < 0.01$) and with A23187 $222 \pm 66 \times 10^9$ L^{-1} ($P > 0.05$).

The activation status of the platelets after stimulation was assessed by measuring their exposure of P-selectin (CD62p), an adhesion receptor present in the α-granule membranes of resting platelets and exposed on the platelet surface upon secretion of the granule contents [29,30], as well as CD63, a member of the tetraspanin superfamily present in lysosomal- and dense granule membranes of resting platelets, and also exposed on the platelet surface upon exocytosis of the respective granules [31,32]. Results are shown in Fig. 1. Compared with inactivated platelets, incubation with calcium alone resulted in higher percentages of platelets exposing P-selectin ($P < 0.001$), but not CD63 ($P > 0.05$). Activation with collagen, thrombin, collagen plus thrombin, or A23187 all resulted in highly increased percentages of platelets exposing P-selectin and CD63 ($P < 0.001$ for all).

Platelet activation also resulted in MP release, representative flow cytometry dot plots of which are shown in Fig. 2 (larger FSC/SSC region: platelets; smaller FSC/SSC region: MPs). Platelets inactivated with dbcAMP hardly released any MPs (Fig. 2A). Platelets treated with calcium alone released slightly more (Fig. 2B), while the various agonists resulted in a pronounced increase in the numbers of MPs released (Fig. 2C–F). The concentrations of MPs released under the various conditions are shown in Fig. 3A. Platelets treated with dbcAMP released only $1.1 \pm 0.3 \times 10^9$ L^{-1} MPs, and platelets incubated with calcium alone released $2.7 \pm 0.6 \times 10^9$ L^{-1} ($P > 0.05$ when compared with dbcAMP-treated platelets). Platelets activated with collagen released $26.9 \pm 5.9 \times 10^9$ L^{-1} MPs ($P < 0.01$), with thrombin $26.5 \pm 21.7 \times 10^9$ L^{-1} ($P < 0.01$), with collagen plus thrombin $50.1 \pm 13.7 \times 10^9$ L^{-1} ($P < 0.001$) and with A23187 $42.2 \pm 14.5 \times 10^9$ L^{-1} ($P < 0.001$).

The exposure of P-selectin and CD63 on the MPs is shown in Fig. 3B. Of the low numbers of MPs released by dbcAMP-treated platelets, 26% ± 11% exposed P-selectin, and 13% ± 5% exposed CD63. Presuming that completely non-activated platelets do not expose these markers of the secretory reaction, these results, and the assessment of activation markers on platelets (Fig. 1), indicate on the one hand that dbcAMP usage does not completely prevent platelet activation, or some preactivation already occurred before the dbcAMP could be added to the initial platelet preparation, and on the other hand that MPs are formed at least partly after the secretory reaction has taken place. Of the MPs

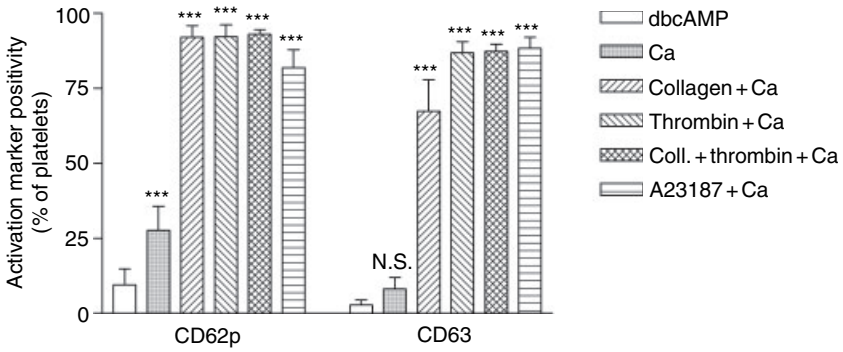

Fig. 1. Exposure of activation markers on the platelets. Exposure of CD62p (P-selectin) and CD63 was measured using flow cytometry on washed platelets ($n = 6$) incubated (i) without a stimulus after pretreatment with dbcAMP, or (ii) with calcium alone, or in combination with (iii) collagen, (iv) thrombin, (v) collagen plus thrombin, or (vi) A23187. Results are expressed as percentage of total platelet numbers and presented as mean ± SD. One-way ANOVA for matched samples was performed, followed by Bonferroni's multiple comparison test between the inactivated (dbcAMP-treated) vs. the other samples. N.S., not significant ($P > 0.05$); ***$P < 0.001$.

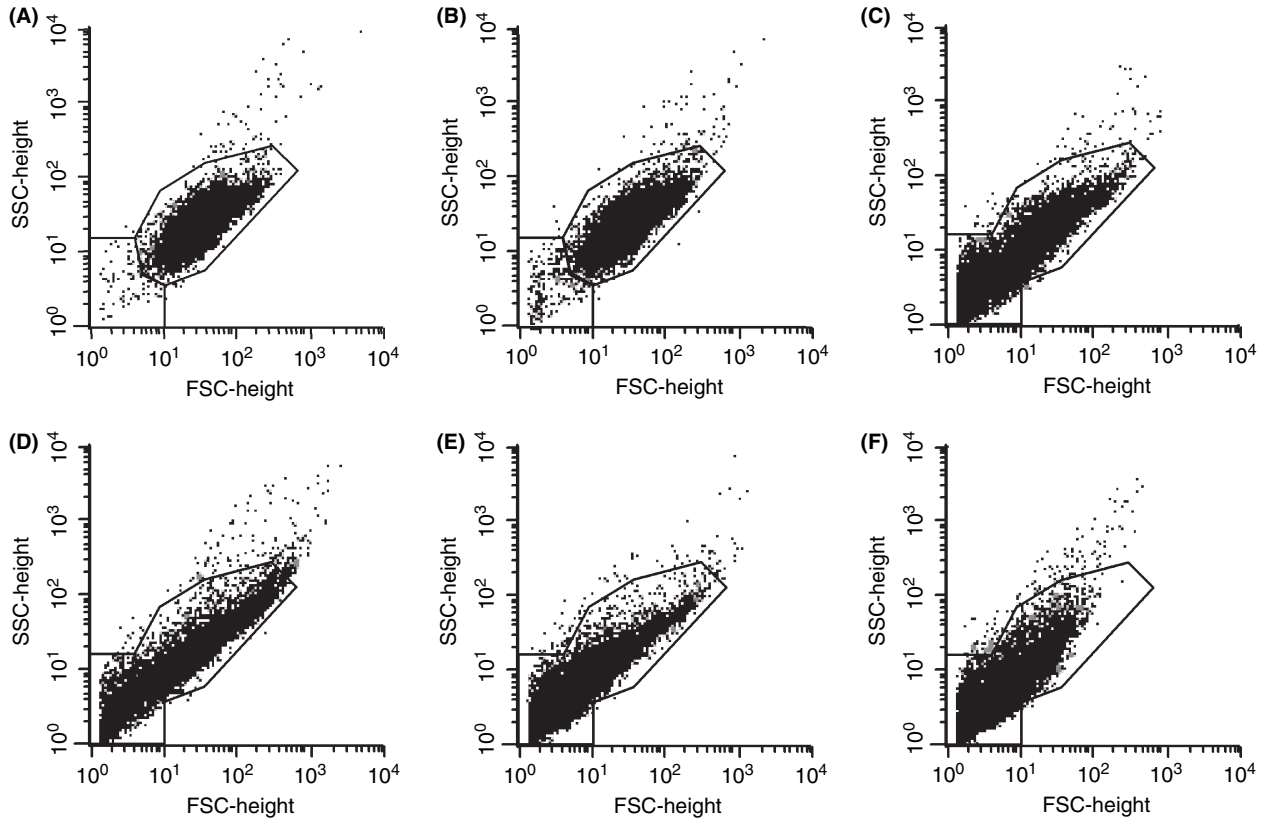

Fig. 2. Flow cytometry dot plots of platelets and MPs released after platelet stimulation. Washed platelets ($n = 6$) were incubated (A) without a stimulus after pretreatment with dbcAMP, or (B) with calcium alone, or in combination with (C) collagen, (D) thrombin, (E) collagen plus thrombin, or (F) A23187, and analyzed using flow cytometry. Representative examples are shown, with FSC vs. SSC dot plots of the platelet marker positive events, and regions drawn around platelets (larger FSC and SSC) and MPs (smaller FSC and SSC).

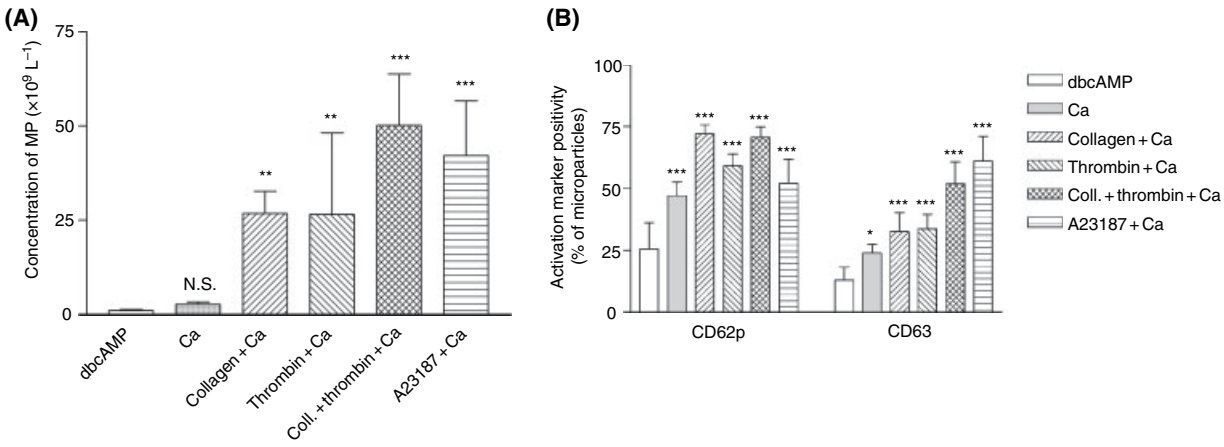

Fig. 3. Microparticles released from stimulated platelets. Washed platelets ($n = 6$) were incubated (i) without a stimulus after pretreatment with dbcAMP, or (ii) with calcium alone, or in combination with (iii) collagen, (iv) thrombin, (v) collagen plus thrombin, or (vi) A23187, and the resulting platelet-MP suspensions were analyzed using flow cytometry. Concentrations of MP released are shown in (A). Exposure of CD62p (P-selectin) and CD63 on the MPs, expressed as percentage of total MP numbers, is shown in (B). Results are presented as mean ± SD. One-way ANOVA for matched samples was performed, followed by Bonferroni's multiple comparison test between the inactivated (dbcAMP-treated) vs. the other samples. N.S., not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

released by platelets incubated with calcium alone, about twice higher percentages exposed these markers (P-selectin: $P < 0.05$; CD63: $P < 0.001$), and regarding the MPs released from platelets activated with collagen, thrombin, collagen plus thrombin, or A23187, these percentages were even higher ($P < 0.001$ for all).

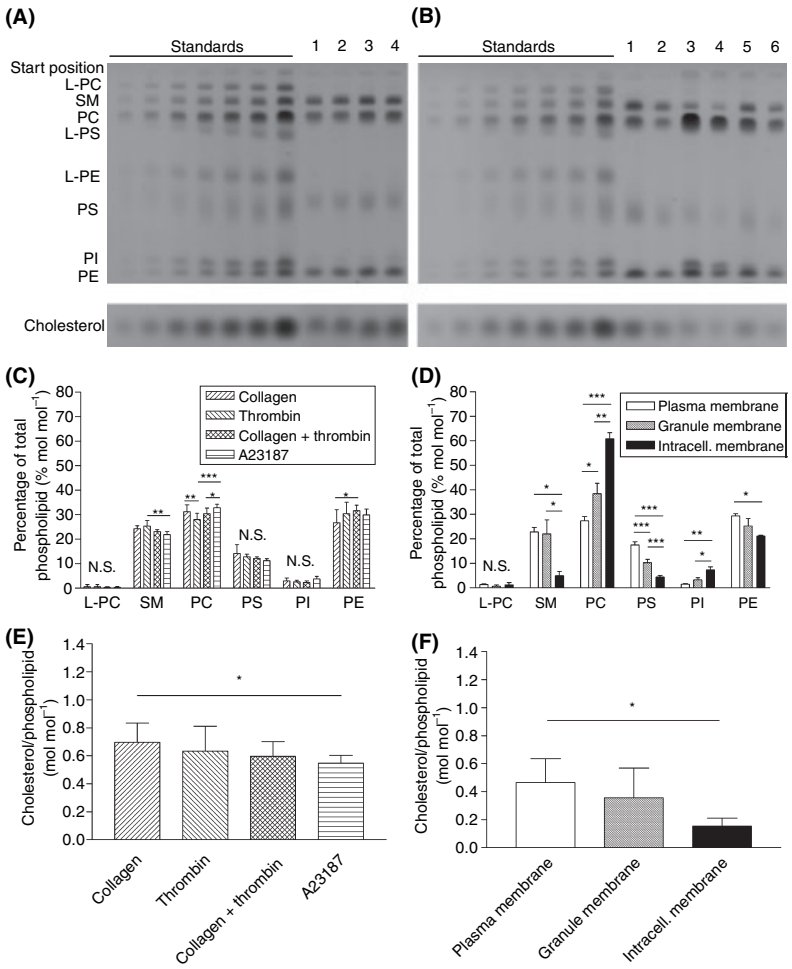

Fig. 4. The phospholipid composition and cholesterol content of platelet-derived MPs and platelet membrane fractions. Washed platelets ($n = 6$) were incubated with collagen and calcium (lane 1), thrombin and calcium (lane 2), collagen plus thrombin and calcium (lane 3), or A23187 and calcium (lane 4). MPs were isolated by differential centrifugation and analyzed using HPTLC for phospholipids and cholesterol. Representative examples are shown in (A). The phospholipid composition of the platelet-derived MPs, expressed as percentage of total phospholipid, is summarized in (C), and the cholesterol/phospholipid molar ratios of the MPs are shown in (E). Platelet plasma- (lanes 1 and 2), intracellular- (lanes 3 and 4) and granule membranes (lanes 5 and 6) were isolated from platelet concentrates ($n = 3$) of 10 donors each, as described in the Methods section. Of each fraction, two different dilutions were analyzed using HPTLC. Representative examples are shown in (B). The phospholipid composition of the platelet membrane fractions, expressed as percentage of total phospholipid, is summarized in (D), and the cholesterol/phospholipid molar ratios of the membrane fractions are shown in (F). To analyze differences between MPs made with different platelet agonists or between platelet membrane fractions, one-way ANOVA for matched samples was performed, followed by Bonferroni's multiple comparison test. The endpoints of the horizontal lines indicate the groups between which significant differences were found. L-PC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; Chol/PL, cholesterol/phospholipid molar ratio; N.S., not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Phospholipid composition and cholesterol content of platelet membrane fractions and platelet-derived MPs

As platelets treated with dbcAMP or calcium alone released only very low numbers of MPs, these had to be excluded from further analysis. MPs obtained with the various platelet agonists were isolated by differential centrifugation, and their lipid contents extracted and analyzed by HPTLC for phospholipids and cholesterol. Representative examples of phospholipid and cholesterol separations on HPTLC plates are shown in Fig. 4A. As shown in Fig. 4C,E, summarizing the quantitative results, there were minor but significant differences between the MPs obtained with different stimuli regarding their SM, PC, PE and cholesterol content. L-PS and -PE were not detected.

For comparison, platelet membrane fractions (plasma-, intracellular and granule membranes) were isolated and also analyzed for phospholipid composition and cholesterol content. Representative examples of the lipid separations are shown in Fig. 4B, and quantitative results are summarized in Fig. 4D,F. As shown in these figures, plasma- and intracellular membranes (the dense tubular system) differed radically in their phospholipid composition and cholesterol content. Plasma membranes contained more SM ($P < 0.05$), PS ($P < 0.001$) and PE ($P < 0.05$) when compared with intracellular membranes, while the latter contained higher

levels of PC ($P < 0.001$) and PI ($P < 0.01$). The cholesterol/phospholipid molar ratio was higher in plasma- than in intracellular membranes ($P < 0.05$). Granule membranes had a phospholipid composition and cholesterol content in between that of plasma- and intracellular membranes. They differed significantly from intracellular membranes in their SM ($P < 0.05$), PC ($P < 0.01$), PS ($P < 0.001$) and PI ($P < 0.05$) content, and from plasma membranes in their PC ($P < 0.05$) and PS ($P < 0.001$) content. L-PS and -PE were not detected.

The phospholipid composition of the MPs was intermediate when compared with that of plasma- and granule membranes, and differed drastically from that of intracellular membranes (Table 1). Compared with plasma membranes, the MPs differed regarding their PS content, and compared with granule membranes, they differed regarding their PC content. Compared with intracellular membranes, MPs differed significantly concerning all phospholipids except L-PC, which was only present in trace amounts in the samples.

Regarding the small but significant differences in phospholipid composition between MPs obtained from platelet suspensions treated with different stimuli, which might be a result of a differing degree of granule membrane contribution to the MP membrane, we examined whether this could be correlated to differences in exposure of protein markers of granule membranes. PC is the phospholipid which differs the most between plasma- and granule membranes, and α-granules constitute the vast majority of platelet granules [15], so we correlated the PC content of MPs with their exposure of P-selectin, a marker of α-granules. Results are shown in Fig. 5. MPs resulting from platelet stimulation with the physiological stimuli collagen, thrombin, and collagen plus thrombin showed a good correlation between these two parameters, while MPs resulting from stimulation with the calcium ionophore A23187 formed a separate population.

The average cholesterol/phospholipid molar ratios of the MPs were higher than those of the three platelet membrane fractions. However, because of the relatively large variations, the differences between MPs and plasma- and granule membranes, except for MPs released from collagen-stimulated platelets, were not statistically significant (Table 1).

Discussion

To gain insight into the mechanism of platelet-derived MP formation regarding the lipid membrane (i.e. lipid sorting), we analyzed the phospholipid composition and cholesterol content of platelet-derived MPs obtained with various stimuli, and compared this to isolated platelet membrane fractions. Our results show that the phospholipid composition of MPs (i) is intermediate compared with that of platelet plasma- and granule membranes, (ii) shows minor but significant differences depending on the platelet agonist and (iii) correlates with the exposure of P-selectin on the MPs in case of the physiological stimuli collagen, thrombin, or a combination of the two. Furthermore, (iv) MPs tended to

Table 1 Differences in phospholipid content and cholesterol/phospholipid molar ratios between MPs made with various stimuli vs. platelet membrane fractions

	Plasma membranes vs.				Granule membranes vs.				Intracell. membranes vs.			
	MP collagen	MP thrombin	MP coll. + thrombin	MP A23187	MP collagen	MP thrombin	MP coll. + thrombin	MP A23187	MP collagen	MP thrombin	MP coll. + thrombin	MP A23187
L-PC	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
SM	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	***	***	***	***
PC	N.S.	N.S.	N.S.	N.S.	**	***	**	N.S.	***	***	***	***
PS	N.S.	*	**	***	N.S.	N.S.	N.S.	N.S.	***	***	***	***
PI	N.S.	N.S.	N.S.	*	N.S.	N.S.	N.S.	N.S.	***	***	***	***
PE	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	*	**	*
Chol/PL	N.S.	N.S.	N.S.	N.S.	*	N.S.	N.S.	N.S.	***	***	**	**

The phospholipid content of the MPs made with various stimuli vs. platelet membrane fractions, expressed as molar percentage of total phospholipid, and the cholesterol/phospholipid molar ratios of these samples were compared with one-way ANOVA, followed by Bonferroni's multiple comparison test.
L-PC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; Chol/PL, cholesterol/phospholipid molar ratio.
MP collagen/thrombin/coll. + thrombin/A23187, MPs released from platelets activated with collagen/thrombin/collagen plus thrombin/A23187 in the presence of calcium. N.S., not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

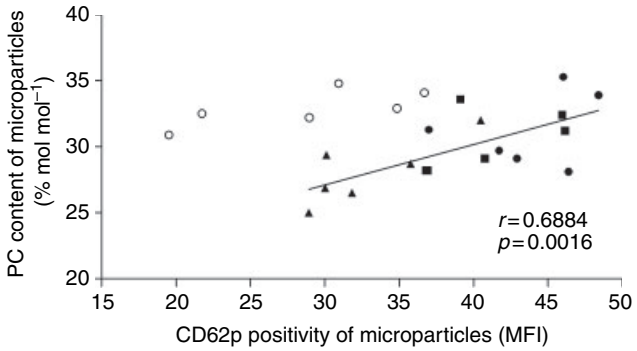

Fig. 5. Microparticle P-selectin (CD62p) exposure vs. PC content. Washed platelets ($n = 6$) were incubated with collagen and calcium (●), with thrombin and calcium (▲), with collagen plus thrombin and calcium (■), or with A23187 and calcium (○). Released MPs were analyzed using flow cytometry for P-selectin exposure and were isolated by differential centrifugation and analyzed using HPTLC for phospholipid content. MP P-selectin exposure [expressed as mean fluorescence intensity (MFI) of the MP population in the flow cytometric analysis] vs. PC content (expressed as molar percentage of total phospholipid) is depicted. Correlation analysis was performed on the MPs obtained by the physiological stimuli collagen (●), thrombin (▲), and collagen plus thrombin (■) using Pearson's correlation test (r, correlation coefficient; P, significance level). The data obtained with A23187 as the platelet agonist (○) were not included as they obviously formed a separate population. If those data were included, $r = 0.1934$ and $P = 0.3652$ would be obtained.

have a higher cholesterol content when compared with platelet membrane fractions, although the differences were statistically not significant.

For isolation of platelet membrane fractions, we used the same procedure as Mauco *et al*. [25] and Fauvel *et al*. [26]. The latter investigators had also analyzed the phospholipid composition and cholesterol content of platelet plasma- and intracellular membranes, and our present results are in excellent agreement with theirs. The lipid composition of granule membranes has so far only been determined by Gogstad *et al*. [33] who isolated platelet α-granules by pressure homogenization of platelets, and centrifugation of the homogenate on a metrizamide density gradient. Our results regarding the lipid composition of granule membranes are comparable with theirs, apart from a higher cholesterol content found in our study. In contrast to our results, however, Gogstad *et al*. reported no significant differences between α-granule membranes and plasma membranes, because of a somewhat different lipid composition they found in plasma membranes isolated by the glycerol lysis technique (higher PC, lower PS + PI), when compared with our present results and those of Fauvel *et al*. [26]. This may be because of the fact that the glycerol lysis technique only results in some 10% of the platelets being lyzed and thus contributing to the membrane preparation.

To generate platelet-derived MPs, we used the platelet agonists collagen, thrombin, collagen plus thrombin, and A23187. The MPs proved to have a phospholipid composition significantly different from that of the intracellular membranes of platelets (the dense tubular system), and intermediate when compared with that of the plasma- and granule membranes. These results suggest that the lipid membrane of the MPs is a composite of platelet plasma- and granule membranes, in line with findings that MPs contain both antigens present on the plasma membrane as well as antigens present exclusively in the granule membranes of resting platelets [4,34,35]. However, apart from the fact that both membrane fractions seem to contribute, our present data do not allow further conclusions to be drawn as to whether MPs originate from membrane areas that are 'richer' in granule membranes than the rest of the platelet surface, or not. The results do indicate that the fusion of the platelet plasma- and α-granule membranes occurs at least partly prior to MP formation.

In 1982, Sandberg *et al*. [36] published a report on 'lipid-protein particles' they isolated from the supernatant of activated platelets. The phospholipid composition they described is comparable to our present results. However, they only used collagen as the platelet agonist. Here, we found small but significant differences in phospholipid composition between MPs produced by different platelet agonists. This suggests that MPs are released in a well-controlled, stimulus-dependent process. Furthermore, based on the relationship between P-selectin exposure and PC content, the calcium ionophore A23187 seems to give rise to MPs that are qualitatively different from MPs made with physiological stimuli. If indeed so, the relevance of results obtained in the past decades using A23187 as a stimulus to obtain MPs can be questioned.

Previously, we reported a somewhat higher PC, lower PS and lower PE content of MPs circulating *in vivo* in healthy individuals [28]. Those results were likely to be influenced by residual plasma present in those preparations. Plasma contains mainly PC and some SM. After subtraction of these, the relative contributions of the other phospholipids increases somewhat, making those results in line with our present data on MPs made *in vitro*. Also, it should be borne in mind that the results previously reported were on total plasma MP preparations, with platelet-derived MPs being the vast majority but not the sole component, e.g. erythrocyte-derived MPs were also present.

Lipid rafts are cholesterol- and sphingolipid-rich membrane microdomains that play an important role in membrane trafficking, protein sorting and signal transduction [37–39]. With such universal functions, it would not be surprising if they were implicated in the process of MP formation as well. However, our knowledge in this area is still quite scarce. Salzer *et al*. [21] demonstrated the presence of lipid rafts in erythrocyte microvesicles, and they found that the lipid raft-associated proteins stomatin and acetylcholinesterase were enriched, while flotillin-1 and -2, also lipid raft-associated proteins, were depleted in the vesicles compared with the erythrocytes. Del Conde *et al*. [40] showed the selective enrichment of tissue factor and P-selectin glycoprotein ligand-1 in microvesicles released from monocytes, both being proteins localized in lipid rafts in the parent cells. Also, cholesterol depletion of a monocytoid cell line decreased microvesicle release, suggesting that vesicle release might be a raft-dependent process. Finally, Mairhofer *et al*. [18] have shown the enrichment of stomatin but not of flotillin-2 in microvesicles released from platelets.

They have also shown that, in platelets, stomatin is mainly localized in the α-granule membranes, only partly associated with lipid rafts, while the flotillins are excluded from α-granules, and are predominantly present in rafts. These results concerning raft-associated proteins in MPs of various cellular origin suggest that rafts, or raft subtypes [18,21], might indeed be implicated in MP formation. However, whether or not the lipids themselves that constitute the rafts (cholesterol, glycosphingolipids, SM) are enriched in the MPs has not been studied in the past. In this study, we found that MPs tended to have a higher cholesterol content when compared with all platelet membrane fractions, though the differences were not significant. Such an increase in cholesterol content of MPs would be in line with the results of Heijnen *et al*. [35] who showed cholesterol accumulation in the filopodia of activated platelets, if one presumes that filopodia give rise to MPs via blebbing [41–43]. On the other hand, we observed no significant differences in SM content in MPs vs. platelet plasma- or granule membranes.

In conclusion, our present findings show that regarding its phospholipid content, the MP membrane is a composite of the plasma- and granule membranes of platelets, showing subtle but significant differences depending on the platelet agonist. The tendency for a higher cholesterol content in the MPs suggests a possible enrichment of lipid rafts in the MPs vs. the (activated) platelet surface, which is a subject of our further investigations.

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