Biochemical properties of platelet microparticle membranes formed during exocytosis resemble organelles more than plasma membrane

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Abstract Studies of [^{3}H]glycerol turnover in phosphatidylcholine (PC) in platelets revealed two metabolic pools, a 'low turnover PC' in collagen-induced microparticles with specific radioactivity only 10% of that found in the 'high turnover PC' of bulk platelet PC. Isolated organelle fractions of [^{3}H]glycerol-labelled platelets contained [^{3}H]PC with specific radioactivities about 20% of that in membrane fractions. These results together with studies on distribution of concanavalin A-FITC and GPIb, a plasma membrane receptor, indicate that microparticles formed during exocytosis are not simple vesiculations of plasma membrane, but they seem rather to originate from a relatively metabolically static membrane pool not accessible to extracellular reagents.

Key words: Platelet microparticle; Phosphatidylcholine turnover; Membrane exocytosis

1. Introduction

Membrane vesicles, referred to as microparticles ([1], review), are shed from blood platelet probably in vivo and definitely in vitro upon aging under blood bank storage [2,3], high shear flow experiments [4] and agonist-induced, regulated exocytosis [5,6]. While microparticle formation induced by age or flow is normally low, agonist-stimulated exocytosis in platelets gives rise to rapid and abundant microparticle formation [5,6]. For abundancy reasons, microparticles formed by exocytosis agonists were used for these studies with the reservation that they may have different biochemical characteristics from those formed by other mechanisms.

Platelet microparticles play an important role in coagulation by providing an extended catalytic surface for binding and activation of specific coagulation factors by surface exposure of phosphatidylserine (PS) [5–15]. Vesiculation or bleb formation on the cell surface accompanied by increased exposure of PS on both platelets and microparticles has been the basis for the accepted view that platelet microparticles are formed by vesicular budding from the plasma membrane of activated platelets.

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In studies of phosphatidylcholine (PC) metabolism in platelets, the incorporation of [^{3}H]glycerol into PC of collagen-induced microparticle membranes was found to be markedly lower than into bulk platelet PC (this article). Platelets lack nuclei and are formed by fragmentation from megakaryocytes as mature cells with prepackaged organelles [16,17] and secretory organelle resynthesis does not occur as in nucleated cells. The low turnover of microparticle membrane PC compared to bulk platelet PC led us to investigate secretory organelle membranes for comparison. The specific radioactivity of [^{3}H]PC after [^{3}H]glycerol labelling was determined in resting platelets, activated platelets, microparticles and isolated secretory organelles. The distributions of a native plasma membrane marker, GPIb, and an extracellular labelling reagent, concanavalin A (ConA), were also studied in resting platelets, in microparticles formed by exocytosis and in subcellular fractions. These studies indicate that exocytosis-associated microparticles or vesicles resemble more closely intracellular organelle membranes than intact platelets, measured as the total platelet content of the various markers.

2. Materials and methods

2.1. [^{3}H]Glycerol labelling and isolation of platelets

Blood was taken by venipuncture from non-medicated, healthy volunteers into 0.15 vol. of ACD, courtesy of the blood bank at Haukeland Hospital. Platelet-rich plasma (PRP) and platelet isolation by gel filtration were carried out as previously described [18]. [^{3}H]Glycerol (1.0 MBq/ml, DuPont NEN, Code NET 848) was added to PRP for 1 h at 22°C before gel filtration or washing by centrifugation in fractionation studies. Calcium-free Tyrode's solution containing 0.2% bovine serum albumin and 5 mM glucose, pH 7.3, was used for gel filtration, pH 6.5 for centrifugation. The platelet counts used were about $4–5\times10^8$/ml.

2.2. ConA labelling of platelets

After [^{3}H]glycerol incubation in PRP, platelets were washed once by sedimentation with calcium-free Tyrode's solution, pH 6.5, resuspended, incubated with 0.5–1 mg of ConA-FITC ((343235), Calbiochem) for 30 min at 22°C and then washed again to remove excess reagent. Concanavalin binding to membrane fractions was measured by fluorescence in 0.1% Triton X-100 (excitation 495 nm, emission 525 nm).

2.3. Activation of labelled platelets

Activation with 20 μg collagen/ml (Kollagen reagens Horm, Nycomed Hormon-Chemie, Munich, Germany) or thrombin receptor agonist peptide (TRAP) 50 μM was carried out at 37°C with stirring (900 rpm) after addition of $CaCl_2$ (4 mM). Aggregation was terminated at 5 min by addition of PGE_1 (25 μM) and the platelets were centrifuged at $13\,000\times g\times4$ min at 4°C and the supernatant saved as the microparticle fraction. Only a negligible pellet which did not detract from bulk supernatant analyses was found after a second centrifugation at $13\,000\times g\times30$ min.

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2.4. Subcellular fractionation of platelets

Washed platelets were treated by metabolic inhibitors to prevent secretion as described previously [19] and then lysed by ultrasonication (50 W on a Branson sonifier B12, microtip probe) for three cycles of 20 s each with 30 s cooling between each cycle. Residual platelets and aggregated debris were removed by centrifugation at $1000\times g\times 10$ min and the organelle fraction sedimented at $10\,000\times g\times 10$ min. The pellet was washed once and the combined supernatants were centrifuged at $100\,000\times g\times 30$ min to give a membrane fraction.

2.5. Lipid extraction and analysis

Total lipid extracts were prepared from 1 ml aliquots of control platelet suspensions, aggregated platelets (resuspended in 1 ml 0.15 M NaCl containing 25 μM PGE_1 or supernatants from collagen-treated platelets as described by Bligh and Dyer [20]. The dried lipids were dissolved in 50 μl chloroform and spotted on aluminum-backed silica platelets (Merck article 5553, DC Alufolien Kieselgel 60). The plates were chromatographed with chloroform/methanol/methylamine 20% in H_2O (60:36:10, v/v). [^{3}H]PC was detected by Radioscanner Isomess 3000 Radio-TLC Analyzer ('RITA', Raytest) or PC mass was visualized by I_2 vapor. The PC spots were scraped, extracted from the silica [21] and aliquots taken for scintillation counting and PC mass determinations as P_i with a malachite green method [22].

2.6. Flow cytometry

Flow cytometric analysis was carried out as previously described [23] with a FACSort flow cytometer (Becton Dickinson, BDIS, San Jose, CA, USA). The platelets were fixed in 0.2% formaldehyde in PBS buffer 2 min after agonist addition; half of each sample was labelled with CD42a-FITC (BDIS) and the other half with chicken anti-human platelet-FITC (Biopool, Umea, Sweden). Gating on both FITC and light scatter profiles identified platelets and microparticles, 5000 events per analysis in triplicate.

3. Results

3.1. [^{3}H]Glycerol turnover in PC in platelets and in microparticles

The specific radioactivity of PC in resting, [^{3}H]glycerol-labelled platelets was 476 ± 155 dpm/nmol PC and 49.4 ± 22 dpm/nmol PC in microparticles from collagen-stimulated platelets (means ± S.D., $n=13$). The mass of PC in five different preparations of microparticles was $20.2\pm12\%$ (S.D.) of that found in resting platelets, comparable to the 15–35% of PF3 (phosphatidylserine) found in microparticles compared to intact platelets reported by Sandberg et al. [6].

3.2. Distribution of ConA in platelets and in microparticles

The amount of concanavalin binding sites and fluorescence label in microparticle membranes was only 6% that of resting platelets, normalized per nmol PC (Table 1).

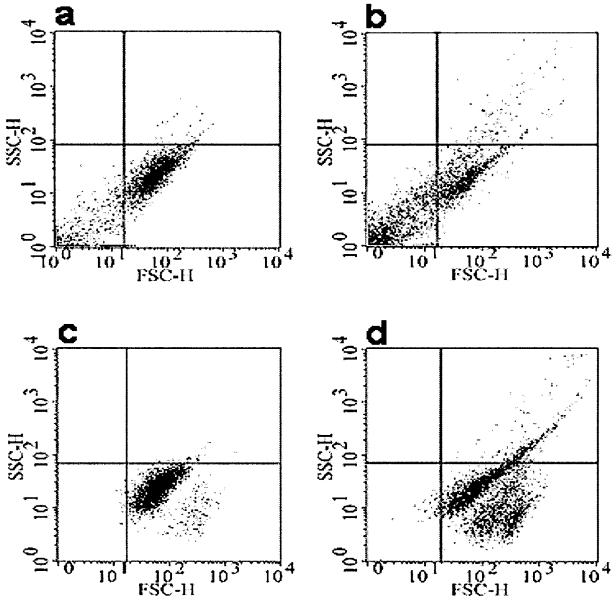

Fig. 1. Flow cytometric analysis of resting and activated platelet suspensions using chicken anti-human platelet antibody and CD42a. See text for description.

3.3. PC specific radioactivity and distribution of ConA in subcellular fractions

[^{3}H]Glycerol-labelled platelets were fractionated as described in Section 2 in order to compare the specific radioactivity of PC in intracellular organelles and in intact platelets (Table 1). An aliquot of the same platelet suspension was stimulated with collagen and the microparticle fraction was analyzed for comparison with organelle membranes. The yield and integrity of the isolated organelles were monitored by assaying for the dense granules which constitute about 50% of total platelet ADP+ATP [19]. The organelle fraction, which includes dense and α-granules and lysosomes, contained $20.9\pm9.3\%$ (S.D., $n=4$) of total platelet ATP/ADP or a 40% yield of dense granules. The concentration of adenine nucleotides (nmol/mg protein) was 9.16 ± 4.4 (S.D., $n=4$) greater than in intact platelets, an 18-fold enrichment of dense granules. The specific radioactivity of the organelle PC was 17% of that of PC in intact platelets. In platelets preincubated with ConA-FITC, the fluorescence per nmol PC of the organelles was 23.8% of that of intact platelets. The latter results

Table 1
The third vertical data column gives the means ± S.D. for five fractionation preparations and the fifth column shows two of these experiments carried out with ConA-FITC

Fraction	Total dpm in PC[a]	Total PC[a] as nmol P_i	PC specific radioactivity dpm/nmol P_i	Total ConA fluorescence[a] (arbitrary U)	ConA-FITC fluorescence per nmol P_i
Intact, resting platelets	39 988	67.9	555 ± 56 ($n=5$)	60.6	0.870, 0.929
Organelles	1 535	17.6	94.4 ± 16 ($n=5$)	3.57	0.203, 0.225
Membranes	25 141	61.9	431 ± 24.5 ($n=5$)	32.4	0.523, 0.730
Microparticles from activated platelets[b]	889	25.1	36 ± 4.1 ($n=4$)	1.38	0.055, 0.053

[a]The total dpm, P_i and fluorescence, columns 1, 2 and 4, respectively, represent data from the first experiment with ConA-FITC shown in column 5. These data were not suited for statistical analysis because the number of platelets and the dpm varied with the preparation although the specific labelling was remarkably similar.
[b]The microparticles were prepared by collagen activation of aliquots from the platelet suspensions that were fractionated and the values in columns 1 and 2 were normalized to be comparable to the same total as the fractionated platelets in the first ConA experiment.

serve as a measure of contamination of the organelle fraction by extracellular membranes because the intracellular organelles would not have been labelled by ConA. The membrane fraction or 100 000 × *g* pellet had a PC specific radioactivity and a ConA-labelling somewhat lower than in intact platelets (Table 1), probably because some surface labelling may have been removed by the nagarse-protease pretreatment used in the fractionation procedure [19].

3.4. Lactate dehydrogenase distribution

The 100 000 × *g* × 30 min pellet (microparticle fraction) from the supernatant of a 1000 × *g* × 10 min preparation of collagen-treated platelets contained about 1.3–1.4% of the lactate dehydrogenase present in intact platelets. Almost no activity was found in the 100 000 × *g* supernatant (cytoplasm plus extracellular medium), indicating that no leakage of cytoplasmic constituents had occurred.

3.5. Distribution of GPIb as studied by flow cytometry

The distribution of a plasma membrane collagen receptor, GPIb, was probed with CD42a and compared with the distribution of a polyclonal antibody raised in chickens to frozen and thawed human platelets that interacts mainly with GPIIb/IIIa (T. Lindahl, personal communication) present in platelet α-granules and plasma membranes [24]. Fig. 1 shows cytometric analysis of resting (a, c) and TRAP-stimulated (b, d) platelets. Panels b and d show the same fixed sample labelled with anti-human platelet-FITC (b) or CD42a-FITC (d). Lower left quadrants show microparticles and intact platelets appear at the right. Microparticles showed almost no CD42a labelling (d).

4. Discussion

Membranes of platelet microparticles formed following collagen or TRAP stimulation of secretion are different from whole platelet membranes and similar to isolated secretory organelles with respect to [^{3}H]glycerol turnover in PC and distribution of ConA in prelabelled cells, microparticles and subcellular organelles (Table 1). Exocytosis-induced microparticle formation occurred with no release of lactate dehydrogenase in contrast to that released with platelet vesiculation during in vitro aging [25].

Flow cytometric experiments have also shown that GPIb which is abundant on the surface of resting and activated platelets was almost not present in agonist-induced microparticles compared to chicken anti-platelet antibody which labels mostly GPIIb/IIIa (Fig. 1). A GPIb-positive, anti-platelet antibody-negative population of particles approximating the size of, but not identical with, platelets labelled with the chicken antibody was inexplicably elevated in TRAP-treated platelets (Fig. 1d). The validity of GPIb as an exclusive plasma membrane marker in platelets is controversial. Although some GPIb appeared to be on granule membranes in histochemical studies [26,27], little was found in isolated organelles compared to GPIIb/IIIa which is present in both α-granules and on the plasma membrane [24]. A recent article reported an even distribution of GPIb on the platelet surface and in the open canalicular system in resting platelets, but no claim was made with regard to GPIb localization on organelle membranes [28]. The work by Zdebska et al. [29] showed that GPIb was present in platelets pelleted at 1000 × *g* and in a 10 000 × *g* × 10 min pellet, but that very much less GPIb/mg protein was seen in smaller particles supports our findings.

The mechanism by which these intracellular membranes reach the outside of intact cells has been problematic, but the recent observations by Wang et al., which show that in vacuole fusion in yeast cells the apposed membranes of the fusing vesicles become detached and free within the new organelle, offer a plausible mechanism [30]. Compound exocytosis, i.e. fusion of secretory organelles at the time of exocytosis, is well-described in platelets as well as sightings of membrane fragments within the fused organelles [30–33]. Release of membrane vesicles, exosomes, from dendrites, T-cells and even platelets has been reported [34]. Heijnen et al. [35] distinguished between microparticles and exosomes by density and by the presence of GPIb on microparticles and its absence on exosomes; although the relative amounts of GPIb on platelets and microparticles were not shown. Microparticles with different membrane properties can be formed by mechanisms other than exocytosis, e.g. complement activation of normal platelets [10] or Glanzmann's thrombasthenia platelets [36] resulted in microparticles profusely labelled with anti-GPIb.

Our results indicate that platelet microparticles in these experiments represent intracellular organelle membranes which serve as a catalytic platform for procoagulant factors. Exocytosis has been considered to be primarily a general mechanism for release of various soluble components, but regulated release of particulate membrane vesicles from cells apparently also takes place and serves as an 'extracellular signalling device' (this article, [34,35,37–39]). However, our results do not discriminate against the possibility that domains of organelle membranes fused to the plasma membrane or other special domains lacking the measured markers might form buds which become detached extracellularly.

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