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Biochemical characterization of plasma membranes and intracellular membranes isolated from human platelets using Percoll gradients

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Two kinds of membranes (plasma membranes and intracellular membranes) have been separated from human platelets by fractionation on Percoll gradients (successively at pH 7.4 and pH 9.6). On alkaline Percoll gradient, plasma membranes floated at low density, as shown with specific markers such as [^{3}H]concanavalin A and monoacylglycerol lipase, whereas intracellular membranes sedimented in the higher densities and displayed a 5.6–12.4-fold enrichment in NADH diaphorase, antimycin insensitive NADH–cytochrome-*c* oxidoreductase and Ca^{2+}-ATPase. Another criterion allowing differentiation of two membrane populations of human platelets was their lipid composition, which showed a cholesterol/phospholipid molar ratio of 0.5 in plasma membranes against 0.2 in intracellular membranes. Phospholipid analysis of the two kinds of membranes displayed also quite different profiles, since phosphatidylcholine increased from 30–32% in the plasma membrane to 52–66% in the intracellular membranes. This was at the expense of sphingomyelin (20–23% in plasma membrane, against 6.8–7.7% in intracellular membranes) and of phosphatidylserine (12–13% in plasma membrane, against 2–6% in intracellular membranes). Other striking differences between plasma membranes and intracellular membranes were obtained by SDS-polyacrylamide gel electrophoresis, which revealed the absence of actin and myosin in the intracellular membrane, whereas both proteins were present in significant amounts in plasma membranes. Finally, intracellular membranes but not plasma membranes were able to incorporate calcium. These results suggest that intracellular membrane fractions are derived from the dense tubular system and plasma membranes should correspond to the whole surface membrane of human platelets.

Introduction

Owing to its specific function in primary hemostasis and coagulation, the surface membrane of platelets is involved in various physiological and biochemical events such as transmembrane signalling, adhesion to different components or cells, exocytosis, and development of a surface catalytic activity able to promote coagulation [1,2]. Although a lot of information about these problems can be gleaned from studies dealing with intact platelets, understanding of their mechanism at a molecular level often requires isolation of the surface membrane in a rather pure form.

Besides the plasma membrane, other intracellu-

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lar membranes play a critical role in the regulation of platelet activation. This is the case for the dense tubular system, which, upon cell lysis, is fragmented into vesicles able to accumulate calcium through an ATP-driven pump [3–7].

Various methods proposed to isolate platelet membranes are generally a compromise between the purity, the yield of membrane material and the length of the separation procedure (for a review, see Ref. 8). A delicate step in purifying platelet plasma membranes consists of their separation from intracellular membranes, which was recently achieved using free-flow electrophoresis by Menashi et al. [9] or by centrifugation on a sucrose cushion by Enouf et al. [10]. We previously described a rapid procedure using self-generating Percoll gradients [11], but our membrane fraction was later found to also contain some other, poorly defined, intracellular membranes [12]. Taking advantage of previous observations made on Krebs II ascites cells [13], a successful separation of plasma membranes from this contaminating fraction could be achieved by centrifugation on alkaline Percoll gradients [12].

This paper deals with the biochemical characterization of two subcellular fractions derived from plasma membrane and dense tubular system, respectively. Evidence of the high purity of these two fractions is based on data from lectin-labelling, marker enzymes, lipid and protein composition as well as on functional studies dealing with calcium uptake. Owing to its versatility and rapidity, such a method could represent an advantageous tool in studies of platelet membrane biochemistry.

Materials and Methods

Materials

Human platelet concentrates obtained from the Blood Transfusion Centre were used within 24 h after blood collection. Alternatively, fresh blood was used immediately after collection onto acid citrate dextrose as previously described [11]. Percoll (poly(vinylpyrollidone)-coated silica particles) was from Pharmacia Fine Chemicals, Uppsala, Sweden. [^{3}H]Concanavalin A (30–90 Ci/mmol) and $^{45}CaCl_2$ (4–50 Ci/g Ca^{2+}) were purchased from Amersham International, Amersham, U.K., and from New England Nuclear, Boston, MA, respectively. Ionophore A23187 was from Boehringer, Mannheim, F.R.G. All other chemicals were obtained from Sigma, Saint Louis, MO, and from Merck, Darmstadt, F.R.G.

Preparation of platelet membrane fractions

Platelets isolated from concentrates or from fresh blood according to Árdlie et al. [14] and suspended in calcium-free Tyrode buffer (pH 6.5) containing 0.2 mM EGTA were incubated with [^{3}H]concanavalin A (100 nCi/ml) for 40 min at room temperature under gentle shaking. After washing with the same buffer lacking EGTA, the cells were suspended in 100 mM KCl, 25 mM Tris-HCl, 3 mM ATP, 3 mM $MgCl_2$ (buffer A) (pH 7.4) and pressurized with nitrogen to 70 atm in a Kontes pressure homogenizer (Kontes, Vineland, NJ) and kept at 0–4°C for 20 min before disintegration. The homogenate was centrifuged at $1500 \times g$ for 10 min at 4°C and the supernatant was collected and pooled for subcellular fractionation as described by Perret et al. [11]: 6 ml of supernatant were mixed with 12 ml of a twice-concentrated buffer A (buffer B) (pH 7.4), 10.32 ml Percoll and 1.68 ml distilled water. The mixture was centrifuged in a Beckman 60 Ti rotor at $79\,000 \times g$ for 15 min and 15 fractions of 2 ml termed 1 to 15 were harvested from the top of the tube. As previously described [11,12], fractions 2 to 4 contained mixed membranes. These fractions (6 ml) were pooled and centrifuged again under the same conditions (pH 7.4) or with a modification of pH. In this case, 10.32 ml of Percoll and 12 ml of buffer B (pH 9.6) were mixed and the pH was adjusted to 9.6 with a few drops of 1 M NaOH. Then 6 ml of mixed membranes were added and the pH was adjusted again exactly to 9.6. The whole procedure was carried out at 4°C. The mixture was centrifuged at $79\,000 \times g$ for 15 min, 15 fractions of 2 ml were collected as described above and were analysed for radioactivity and enzyme markers. In other cases, fractions 2 to 4 of the Percoll gradient pH 7.4 (mixed membranes), fractions 2 to 4 of the Percoll gradient pH 9.6 (plasma membranes) and fractions 13 to 15 of the Percoll gradient pH 9.6 (intracellular membranes) were diluted 3-fold with buffer A (pH 7.4) and sonicated for twice 30 s using an MSE sonica-

tor at maximum output. After centrifugation at 200 000 × *g* for 60 min, each membrane fraction was resuspended in a minimum volume of buffer A (pH 7.4) for further determinations.

Enzyme assays

NADH diaphorase (recommended name NADH dehydrogenase), also called NADH : ferricyanide reductase (EC 1.6.99.3), was measured according to a modified procedure of Wallach and Kamat [15] described by Record et al. [13], with equal concentrations (0.7 mM) of NADH and ferricyanide in 100 mM Tris-HCl buffer (pH 7.4) and various protein concentrations in a final volume of 1 ml. The enzyme activity was followed by ferricyanide reduction at 410 nm.

The same enzyme (EC 1.6.99.3) using cytochrome *c* as an acceptor in the presence of antimycin (antimycin-insensitive NADH–cytochrome-*c* reductase) was assayed as described by Tolbert [16]. The 2.7 ml final volume mixture contained 200 mM phosphate buffer pH 7.0, 0.07 mM oxidized cytochrome *c*, 0.19 mM potassium cyanide and 0.02 ml antimycin A (2 mg/ml). The reaction was initiated with 0.8 mM NADH and various protein concentrations and was followed at 550 nm for 10 min.

N-Acetyl-β-D-glucosaminidase (EC 3.2.1.30) was detected by hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (1.7 mM) in 100 mM citrate buffer (pH 4.2), according to Day et al. [17].

Ca^{2+}-ATPase activity: for determination of Ca^{2+}-ATPase, the following reaction mixture was used: 25 mM Tris-HCl (pH 7.4), 100 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 0.05 mM $CaCl_2$ and different protein fractions in a final volume of 0.5 ml. After incubation at 25°C for 30 min (or 60 min), 0.05 ml of the medium was added to 2 ml malachite green reagent used for the colorimetric determination of inorganic phosphorus [18,19]. The Ca^{2+}-dependent ATPase activity was calculated by subtracting the ATPase activity measured in the presence of 5 mM EGTA and without adding $CaCl_2$ from that obtained with added $CaCl_2$. Results are expressed as nmol inorganic phosphorus liberated per min per mg protein.

1(3)-Acyl-*sn*-glycerol lipase and 2-acyl-*sn*-glycerol lipase activities: radioactive 1-monoolein (99.8% 1(3)-isomer, 0.2% 2-isomer) and 2-monoolein (95.9% 2-isomer, 4.1% 1(3)-isomer) (607 dpm/nmol) were purified by thin-layer chromatography on Silica gel developed in benzene/ethyl acetate/trimethyl borate (100 : 20 : 7.2, v/v) [20,21]. Lipid substrates were taken to dryness under nitrogen and dispersed by vortex agitation in a minimal volume of ethanol and 5% fatty acid-free bovine serum albumin. The incubation medium contained, in a final volume of 0.2 ml, 0.5 mM monoacylglycerol substrate, various concentrations of proteins in 50 mM piperazine/glycyl glycine buffer (pH 6.2) containing 10 mM $CaCl_2$. After incubation at 37°C for 30 min under shaking, radioactive free fatty acids were extracted according to Belfrage and Vaughan [22] as described by Mauco et al. [12].

Extraction and estimation of lipid contents

Lipids were extracted according to Bligh and Dyer [23] or Reed et al. [24] (first extraction under neutral conditions and then acidic conditions) in the presence of 16 mM EDTA. Cholesterol was determined by gas-liquid chromatography on a capillary column (5 m × 0.31 mm i.d.) packed with cross-linked methylsilicone (SP 2100) (Intersmat analyzer, model IGC 120 DFL) using 5α-cholestane as internal standard [25] and phospholipid phosphorus was measured according to Böttcher et al. [26]. Phospholipid composition was determined by two-dimensional thin-layer chromatography on Silica gel-coated plates using the solvent system of Esko and Raetz [27]. The different spots were scraped off and analysed by their phosphorus content according to Böttcher et al. [26].

Assay of calcium uptake

Calcium uptake was assayed essentially according to Robblee et al. [4]. The incubation mixture contained 100 mM KCl, 25 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 3 mM ATP buffer, 1 mM potassium oxalate, 0.05 mM $^{45}CaCl_2$ (specific radioactivity 56 nCi/nmol) and various vesicle or membrane concentrations. Incubations were performed at 25°C. At various times, 0.2 ml aliquots were withdrawn and immediately filtered through an Amicon sampling unit using 0.5 μm pore size filters (VFM 3 apparatus). After washing by 30 ml 9% KCl (w/v), the filters were air-dried, solubi-

lized in 10 ml Aquasol and the radioactivity was determined. For calcium release experiments, A23187 dissolved in dimethyl sulfoxide was added under a minimal volume (0.005 ml/ml) after 60 min incubation and the radioactivity remaining in the vesicles was determined as above. Control experiments were performed in parallel after adding the same volume of dimethyl sulfoxide.

SDS-polyacrylamide gel electrophoresis

Membranes were solubilized in 2% (w/v) SDS, 5% (w/v) 2β-mercaptoethanol at 100°C for 3 min. SDS-polyacryl slab gels (3% stacking gel, 7% resolving gel) were prepared and run in a buffer system of 0.025 M Tris-HCl, 0.192 M glycine (pH 8.3) containing 0.1% SDS [28]. Bromophenol blue was used as a tracking dye. Gels were run at 120 V in an LKB apparatus (Vertical Electrophoresis Unit 2001) until the tracking dye reached the bottom of the gel. Silver staining of the proteins was performed by the procedure described by Morrisey [29].

Analytical methods

Radioactivity was determined in a liquid scintillation photometer (Intertechnique, model 4000) equipped with automatic quenching correction. Protein was determined by the method of Lowry et al. [30], using bovine serum albumin as a standard or by the method of Khan et al. [31] when Percoll was present in the preparation.

Results

Separation of platelet plasma membrane from intracellular membranes

As previously described [11,12], two major fractions appeared after centrifugation of the 1500 × *g* supernatant on a Percoll gradient at pH 7.4. The low-density band at the top of the tubes contained the plasma membrane markers, [^{3}H]concanavalin A and bis(*p*-nitrophenyl) phosphodiesterase [11] as well as the intracellular membrane marker NADH diaphorase [12]: this will be referred to as the mixed membrane fraction. The high-density band was previously found enriched in *N*-acetyl-β-D-glucosaminidase and [^{14}C]serotonin [12], and this consisted of various granules.

Upon recentrifugation of the mixed membrane fraction on Percoll at pH 7.4, a single band was again observed at the same position, whereas two different bands appeared at low and high density, respectively, when pH was increased to 9.6. As illustrated in Fig. 1B, the upper low-density band was specifically labelled with [^{3}H]concanavalin A, whereas the lower high-density band corresponded to a peak of NADH diaphorase. In contrast, both markers focused together at low density on Percoll gradients at pH 7.4 (Fig. 1A). This fractionation procedure was thus found suitable for separating in a relatively quick way plasma membranes and intracellular membranes. Under these conditions, proteins recovered in plasma membranes and in-

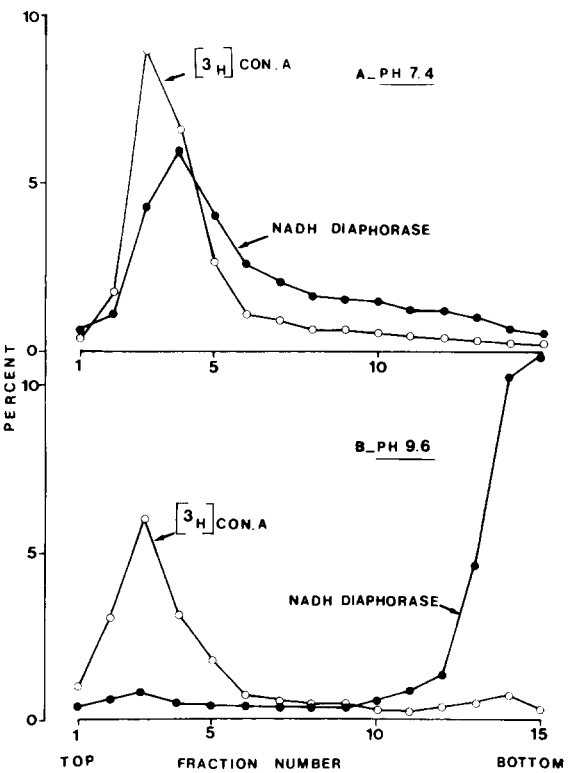

Fig. 1. Subcellular fractionation of platelet homogenate on Percoll gradients. The 1500 × *g* supernatants from disrupted platelets were mixed with Percoll (34%, v/v) at pH 7.4 and centrifuged for 15 min at 79000 × *g*. Mixed membrane fractions were pooled and centrifuged again with Percoll (34%, v/v) at pH 7.4 (A) and at pH 9.6 (B). In both cases, 15 fractions of 2 ml were collected from the top of the tube. The activity of NADH diaphorase (●———●) and the binding of [^{3}H]concanavalin A (○———○) were determined in each fraction and expressed as percentages of the homogenate. Conditions are those described in Materials and Methods.

TABLE 1

SPECIFIC ACTIVITY AND RADIOACTIVITY OF VARIOUS MARKER ENZYMES

Mixed membranes, plasma membranes and intracellular membranes were obtained after dilution with buffer A and centrifugation at 200000 × *g* for 60 min for the elimination of Percoll. Various markers were determined as described in Materials and Methods. Results are given as means (range values) from three experiments (except for 2-MG-lipase, two experiments). Numbers in the second parentheses represent the enrichment ratio. Abbreviation: MG, monoacylglycerol.

	Homogenate	Mixed membranes	Plasma membranes	Intracellular membranes
[^{3}H]Concanavalin A (dpm/mg protein)	6293 (4129, 8132)	15339 (7183, 21950) (2.4)	39292 (22264, 56760) (6.2)	5837 (1681, 8055) (0.92)
1-MG-lipase (nmol/min per mg protein)	15.5 (11.4, 17.5)	66.7 (58.0, 77.0) (4.3)	76.4 (62.3, 113.1) (4.9)	18.45 (16.0, 26.9) (1.10)
2-MG-lipase (nmol/min per mg protein)	15.5 (14.8, 16.2)	35.7 (35.7, 35.7) (2.3)	38.7 (38.0, 39.4) (2.5)	16.40 (16.2, 16.6) (1.00)
NADH diaphorase (nmol/min per mg protein)	344 (283, 400)	508 (345, 699) (1.5)	466 (231, 748) (1.4)	4141 (2468, 7267) (12.40)
Glucosaminidase (μmol/h per mg protein)	268 (217, 296)	193 (185, 200) (0.7)	264 (198, 308) (0.99)	177 (119, 293) (0.68)
Antimycin-insensitive NADH–cytochrome *c* oxidoreductase (nmol/min per mg protein)	2.2 (1.8, 2.9)	2.2 (1.71, 2.61) (1.0)	2.6 (1.6, 3.8) (1.2)	11.1 (8.7, 14.1) (5.60)
Ca^{2+}-ATPase (nmol P_i/min per mg protein)	10 (9.2, 10.9)	6.9 (4.3, 10.0) (0.8)	8.6 (4.6, 14.6) (1.0)	91.5 (70.3, 108.3) (10.3)

tracellular membranes ranged between 1 and 1.5 mg and 0.3 and 0.5 mg, respectively, starting from two platelet concentrates. This corresponded to a recovery of 16% of [^{3}H]concanavalin A in plasma membranes and 12% of NADH diaphorase in intracellular membranes.

Enrichment of various markers in the different membrane fractions

Table I reports the specific activities of various markers in the different membrane fractions. [^{3}H]Concanavalin A, 1(3)-acyl-*sn*-glycerol lipase and 2-acyl-*sn*-glycerol lipase were found to be enriched 2.3–4.3-fold in mixed membranes and 2.5–6.2-fold in plasma membranes, versus 0.92–1.1-fold in intracellular membranes. In contrast, enrichment ratios of 5.6, 12.4 and 10.3 were observed in intracellular membranes for NADH–cytochrome-*c* oxidoreductase, NADH diaphorase and Ca^{2+}-ATPase, respectively. The enrichment ratio of these three enzymes was lowered to 0.8–1.5 in both mixed membranes and plasma membranes. As for *N*-acetyl-β-D-glucosaminidase, this lysosomal marker did not display any enrichment in any of the fractions tested.

Lipid composition

Table II reports the total phospholipid and cholesterol content of homogenate, plasma membranes and intracellular membranes. Phospholipids were enriched 4.7-times and 3.5-times in plasma membranes and intracellular membranes, respectively, versus 5.9 and 1.70-times for cholesterol. As a consequence, the cholesterol/phospholipid molar ratio was increased from 0.41 to 0.52 between homogenate and plasma membranes and was lowered to 0.19 in intracellular membranes.

Other striking differences between plasma membranes and intracellular membranes appeared when considering their phospholipid composition. Indeed, as shown in Table IIIA, plasma membranes were enriched in both sphingomyelin and phosphatidylserine at the expense of phosphatidylcholine, which was lowered from 43.6% to 32.6%. The latter phospholipid was dramatically increased in intracellular membranes (over 50% of total phospholipid), whereas sphingomyelin and phosphatidylserine were decreased to 6.8% and 2.3%, respectively (versus 20.5% and 13.3%, respectively, in plasma membranes).

A surprising finding was the low content of phosphatidylinositol in plasma membranes isolated from platelet concentrates (0.7%). Since phosphatidylinositol was only 1.4% of total cell phospholipids, versus 4–6% from various literature data [11,32,33], this promptedus to perform additional experiments using fresh blood. As shown in Table IIIB, in this case, phosphatidylinositol content was 4.4% for total platelets, versus 3.2% for plasma membranes and 7.4% for intracellular membranes. No major changes compared to the data of Table IIIA were observed for the other phospholipids. These experiments strongly suggest that a significant hydrolysis of phosphatidylinositol occurs during the preparation and handling of platelet concentrates and might well correspond to some activation of platelets.

SDS-polyacrylamide gel electrophoresis

The protein profiles obtained with mixed membranes, plasma membranes and intracellular mem-

TABLE II

LIPID COMPOSITION OF PLATELET SUBFRACTIONS

Platelet membranes are prepared as described in Table I. Values are expressed as means (range values) from three experiments.

	Homogenate	Plasma membranes	Intracellular membranes
Phospholipids (μmol/mg protein)	0.229 (0.197, 0.250)	1.08 (0.990, 1.143)	0.797 (0.660, 0.930)
Cholesterol (μmol/mg protein)	0.094 (0.092, 0.095)	0.553 (0.492, 0.623)	0.157 (0.102, 0.212)
Cholesterol/phospholipids (molar ratio)	0.41	0.52	0.19

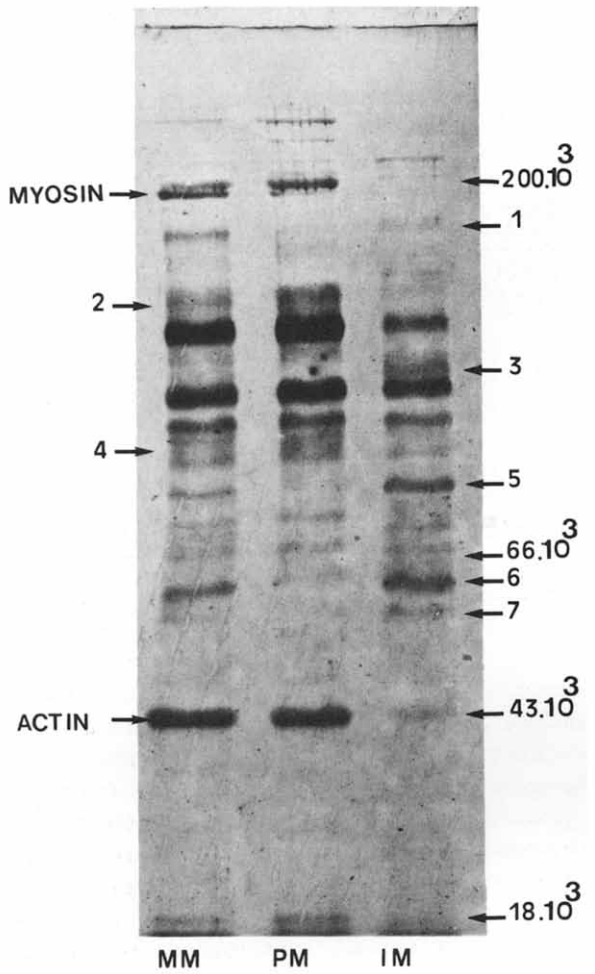

Fig. 2. SDS-polyacrylamide gel (7%) separation of platelet membrane fractions after silver-staining procedure. MM, mixed membranes; PM, plasma membranes; IM, intracellular membranes. Each fraction contains 0.010 mg of total proteins.

branes are given in Fig. 2. Under our experimental conditions, mixed membranes were shown to contain over 25 different bands, among which myosin and actin could be easily identified. Both proteins were also found in plasma membranes, whereas they disappeared or were hardly detectable in intracellular membranes. Among other unidentified components, bands 2 and 4, as indicated in Fig. 3, were exclusively present in mixed membranes and plasma membranes. In contrast, bands 1, 3, 5, 6 and 7 were only detected in mixed membranes and intracellular membranes.

Calcium uptake

In the presence of 1 mM oxalate and 3 mM ATP, mixed membranes were able to accumulate $^{45}Ca^{2+}$ at a rather low level (2 nmol/h per mg protein). As shown in Fig. 3A, this was still lower with plasma membranes, whereas intracellular

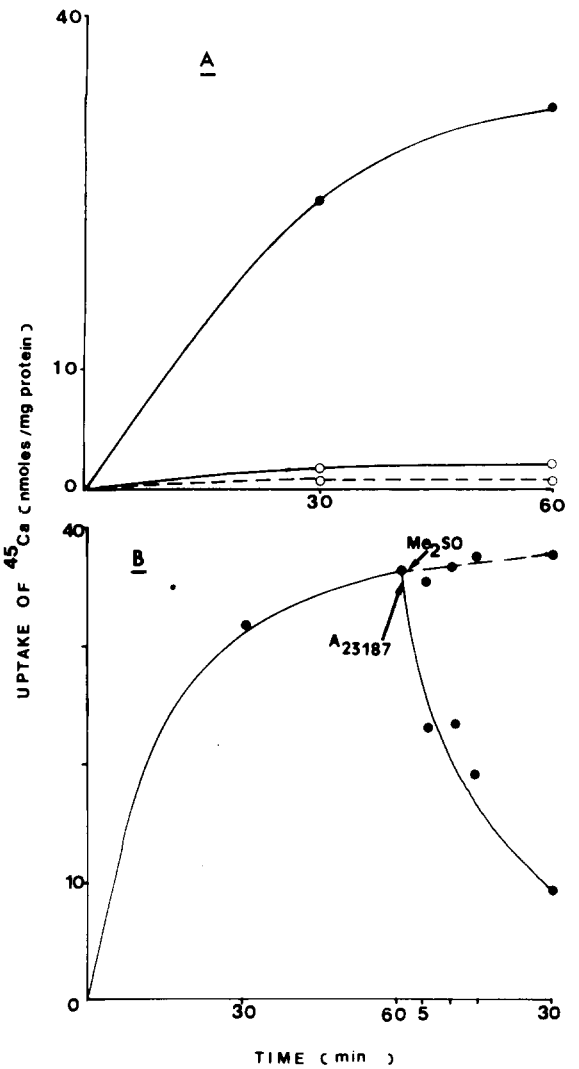

Fig. 3. A. Uptake of calcium by the mixed membrane fraction (○——○), the plasma membrane fraction (○ — — — ○) and the intracellular membrane fraction (●——●). Ca^{2+} uptake was measured in the presence of 3 mM ATP, 1 mM oxalate and 0.05 mM $CaCl_2$ (^{45}Ca: 56 nCi/nmol). B. Release of Ca^{2+} from preloaded intracellular membrane vesicles by ionophore A23187 (5 μM). Ca^{2+} uptake was measured under conditions described in (A). Me_2SO, dimethyl sulfoxide.

TABLE III

PHOSPHOLIPID COMPOSITION OF PLATELET HOMOGENATE AND SUBFRACTIONS

Membrane subfractions were prepared, A, from platelet concentrates, or B, from platelets of fresh blood. Individual phospholipids were isolated by two-dimensional thin-layer chromatography as described in Materials and Methods and expressed as percentage of total phospholipid phosphorus. Results are the means (range values) of three experiments for A and of two determinations for B.

	Homogenate	Plasma membranes	Intracellular membranes
A			
Sphingomyelin	15.98 (15.3, 16.8)	20.48 (19.0, 22.7)	6.80 (3.3, 11.0)
Phosphatidylcholine	43.63 (42.3, 44.4)	32.58 (31.8, 35.5)	66.30 (56.4, 70.0)
Phosphatidylserine	8.30 (7.0, 9.7)	13.25 (12.0, 13.7)	2.28 (1.5, 4.0)
Phosphatidylinositol	1.43 (1.5, 2.6)	0.73 (0.4, 0.9)	3.28 (1.6, 4.1)
Phosphatidylethanolamine	30.57 (30.1, 30.9)	32.85 (31.9, 35.0)	21.25 (16.0, 24.5)
B			
Sphingomyelin	18.60 (18.1, 19.0)	23.00 (21.4, 24.6)	7.70 (7.5, 7.8)
Phosphatidylcholine	38.40 (36.5, 40.3)	30.60 (29.2, 32.0)	52.50 (51.5, 53.4)
Phosphatidylserine	10.40 (10.4, 10.4)	11.90 (10.1, 13.6)	6.00 (5.2, 6.7)
Phosphatidylinositol	4.40 (3.9, 4.8)	3.20 (2.8, 3.6)	7.40 (6.9, 7.9)
Phosphatidylethanolamine	28.50 (27.4, 29.5)	31.30 (30.2, 32.4)	26.50 (26.1, 26.9)

membranes displayed a 10-fold higher activity compared to plasma membranes. Evidence that calcium was accumulated inside vesicular structures was obtained from the observation that A23187 was able to release over 50% of the cation within 5 min (Fig. 3B).

Discussion

The present study allowed us to perform a biochemical and functional characterization of two membrane fractions which are believed to derive from platelet plasma membranes and from dense tubular system. The simple and quick procedure used for such a separation relies on a striking effect of pH towards the apparent density of intracellular membranes. We previously took advantage of this poorly understood effect to isolate plasma membranes from Krebs ascites cells [13] and from human polymorphonuclear leukocytes [34]. Also the platelet diacylglycerol lipase potentially involved in arachidonate liberation was unambiguously localized in plasma membranes by using a similar separation procedure [12]. This has been recently confirmed by Authy et al. [35] using saturating concentrations of diacylglycerol.

Various data presented in this paper favor the view that the plasma membrane fraction is indeed derived from platelet surface membrane. These include a 5-fold enrichment in [^{3}H]concanavalin A, the presence of actin and myosin, and a high cholesterol/phospholipid ratio, as well as an increased content of sphingomyelin and phosphatidylserine at the expense of phosphatidylcholine. This peculiar lipid composition has already been used as a characteristic feature of plasma membranes from various cells, including platelets [36–38]. As to the enrichment ratio of [^{3}H]concanavalin A, Sixma and Lips [8] previously proposed on the basis of various arguments that this should correspond to a maximal value as far as platelets are concerned. It is interesting to note that monoacylglycerol lipase displays the same enrichment as [^{3}H]concanavalin A, suggesting this enzyme as a convenient marker of human platelet plasma membrane.

Unfortunately, such a good degree of purity is obtained at the expense of the overall yield of membrane material (16% and 12% for [^{3}H]concanavalin A and NADH diaphorase, respectively). A first loss of membrane material (22% of [^{3}H]concanavalin A and 45% of NADH dehydrogenase) occurs in the $1500 \times g$ centrifugation (Mauco, G., et al., unpublished data). However, this appeared as a necessary step, since it allowed elimination of partially lysed platelets, which dis-

play the same density as mixed membranes on the Percoll gradient [11]. Further loss occurs in the first Percoll gradient by selecting fractions 2 to 4, which do not contain the whole mixed membrane peak. It should also be recalled that 40% of the NADH diaphorase marker focuses in the granule fraction in the first Percoll gradient [12]. We do not know at the present time whether this heavier fraction corresponds to another population of vesicles derived from the dense tubular system or whether NADH diaphorase is not also present in granules or mitochondria. Owing to these various reasons, we previously reported a recovery of 26% for [^{3}H]concanavalin A and 19% for NADH diaphorase in the mixed membrane fraction [12]. So, further loss during the second Percoll gradient at pH 9.6 can simply be explained by selection of the most active fractions of each membrane peak.

Using a quite different procedure based on neuraminidase treatment of intact platelets followed by free-flow electrophoresis of mixed membranes isolated by centrifugation on sorbitol gradient, Menashi et al. [9] succeeded in isolating two surface membrane fractions displaying a purity similar to that reported in the present study. They suggested that the two fractions could originate from different domains in the surface membrane, i.e. the external surface and the open canalicular system. However, no significant difference in lipid and protein composition or marker distribution appeared between those two fractions. Thus, it seems reasonable to conclude that the plasma membrane fraction obtained using our procedure should correspond to the whole surface membrane.

The intracellular membrane fraction, which shifted to higher densities at alkaline pH, displays the main characteristics previously described by Menashi et a. [9] for fraction NI isolated by free-flow electrophoresis: lack of [^{3}H]concanavalin A labelling, absence of actin and myosin, enrichment in NADH–cytochrome-*c* reductase, low cholesterol content. Also the specific decrease of sphingomyelin at the expense of phosphatidylcholine is a general feature of intracellular membranes, as shown for platelets [38], rat liver endoplasmic reticulum [36] and muscle sarcoplasmic reticulum [39]. In addition to NADH–cytochrome-*c* reductase, NADH : ferricyanide reductase also appears as a useful marker for platelet intracellular membranes and has already been successfully used in Krebs ascitic cells [13] and human polymorphonuclear leukocytes [34], where it displays the same distribution as CDP choline : diacylglycerol choline-phosphotransferase, a specific enzyme of the endoplasmic reticulum.

The high calcium-ATPase activity measured in the intracellular membrane fraction is probably further evidence of the calcium pumping activity detected using ^{45}Ca uptake. Such a property has already been reported by various groups [3–7], who suggested that it corresponded with membrane vesicles derived from the platelet-dense tubular system. However, a clear separation of this fraction from platelet surface membrane was only achieved by Menashi et al. [40–41] using free-flow electrophoresis and by Enouf et al. [10] using centrifugation on a sucrose cushion. Recently, O'Rourke et al. [42] isolated on a neutral Percoll gradient, very similar to that used in the first step of our procedure, a vesicle fraction able to actively sequester calcium. Although data on [^{3}H]concanavalin A did not appear in their gradient profile, their comments indicated that plasma membranes focused at a very close, somewhat lower density, probably similar if not identical to the situation presented in Fig. 1A. So, the characterization of this fraction performed in the present study gives further support to the conclusion of O'Rourke et al. [42] that the platelet-dense tubular system is the target of inositol 1,4,5-trisphosphate as an intracellular calcium-mobilizing agent.

Our failure to detect a Ca^{2+}-ATPase as well as a Ca^{2+} pumping activity in the plasma membrane fraction is similar to that of Menashi et al. [40] and Steiner and Lüscher [43]. This does not necessarily mean that Ca^{2+}-ATPase is lacking in the platelet plasma membrane, since neuraminidase treatment [40] or alkaline pH (this study) might have been deleterious to the enzyme. Special conditions such as omission of magnesium can also be required to detect such an enzyme in certain plasma membranes, as in hepatocytes [44]. Nor can we rule out the possibility that plasma membranes were resealed in an inadequate orientation (right side out), rendering the substrate inaccessible to the enzyme. This is probably not the case, since our mixed membrane fraction was recently found to phosphorylate proteins and inositol-phospholipids

upon incubation with [γ-^{32}P]ATP [45]. In this respect, it is important to mention that Enouf et al. [10] detected an active calcium pump in isolated human platelet plasma membranes. However, their platelet lysates were prepared in the presence of potassium oxalate during sonication, which might have led to entrapment of the organic anion in inside out vesicles. But further studies are still required to explain such a discrepancy.

Taken together, our present data clearly indicate that we succeeded in isolating from human platelets two purified fractions, probably corresponding to surface membrane and dense tubular system, respectively. Besides its rapidity, our method presents the advantage of versatility, since it requires no special equipment. So this procedure should help in developing studies dealing with a better characterization of platelet membrane proteins and enzymes, calcium movements and their regulation, and phospholipid metabolism.

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