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Unequal distribution of membrane components between pseudopodia and cell bodies of platelets

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Platelet pseudopodia were compared to platelet cell bodies with respect to their lipid composition, fatty acid distribution and protein composition. The methodology for producing pseudopodial preparations of platelets stimulated with thrombin, ADP or calcium ionophore was established. The separation of pseudopodia and cell bodies was verified by electron microscopic examination of the respective platelet components. Lipid analyses demonstrated a preponderance of lysophospholipids and sphingomyelin in pseudopodial preparations and a large increase in mono-, di- and tri-ene fatty acids as compared to cell bodies. Changes were also evident in the protein composition evaluated by one- and two-dimensional SDS-polyacrylamide gel electrophoresis and by [^{32}P]ATP labeling of exofacial membrane proteins. A protein of approximately 68 kDa which reacted strongly with antibody to PI^{A1}, was prominantly displayed in platelet pseudopodia. Thus, our studies demonstrate a heterogeneous distribution of lipids and proteins in a mammalian membrane system which may have important implications for the functional behavior of the cell.

Introduction

Membranes not only compartmentalize the cell but also are the site of important enzyme activities, transport phenomena and receptors for hormones and other modifiers of cell function. The chemical composition and the physical state of the lipids in the membrane are of crucial importance for the function and properties of the membrane systems. Although most of the available techniques reveal the lipid matrix of membranes to be a miscible continuum in the physiologic temperature range of mammalian organisms, there is considerable evidence from a variety of sources that such a continuum may in fact be disrupted by discrete areas of lipid heterogeneity [1–9]. But neither the existence nor the functional role of lateral phase separations has been unequivocally demonstrated. Most of the methods used to evaluate membrane behavior measure bulk properties unable to yield data on discrete areas of the plasma membrane.

Platelets are uniquely suited to test whether areas of distinct lipid composition can be maintained for extended periods of time in membrane systems. Upon stimulation by agonists, platelets rapidly undergo a change in shape from a discoid form to a sphere with pseudopodial processes. This morphologic change occurs on a time scale of seconds following the interaction of agonists with their respective receptors on the surface of the platelet [10]. The extended rather rigid filaments that protrude from the platelet body can be easily separated by moderate shear force. In addition, platelet stimulation without stirring by certain complement proteins and a variety of agonists has been shown to lead to the release of small membrane vesicles that have unique compositional characteristics [11–14].

Our studies were prompted by the discovery that nutritional supplementation with alpha-tocopherol inhibits the later stages of agonist induced pseudopodia formation in platelets [15]. We report here on the changes in lipid composition, fatty acid distribution, and protein composition of platelet cell bodies and platelet pseudopodia. Our studies reveal a preponderance of lysophospholipids in the pseudopodial preparations and a large increase in mono-, di- and tri-ene fatty acids in pseudopodia as compared to cell bodies. There is also a striking difference in the protein composition. Platelet pseudopodia prominently display a protein which is found only in low abundance in cell body preparations. [^{32}P]ATP labeling of exofacial membrane

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proteins gave further evidence of unequal distribution of such proteins. Our studies thus provide demonstration of heterogeneous distribution of lipids in a mammalian membrane system which has important implications for the functional behavior of these cells.

Methods and Materials

Preparation of platelets and isolation of platelet pseudopodia

Platelets were isolated from fresh blood obtained from volunteer donors who had abstained from any medication affecting platelet function for a minimum of 10 days prior to phlebotomy or from platelet concentrates prepared and supplied by the Rhode Island Blood Center. The platelets from 2–3 units of blood were pooled, contaminating red blood cells eliminated by brief centrifugation at $580 \times g$ and platelets were pelleted after addition of 15 vol% ACD to the platelet-rich plasma [16]. After washing twice in 3 mM (*N*-morpholino)ethanesulfonic acid containing 0.147 M NaCl, 3 mM KCl and 4.8 mM D-glucose (Mes buffer), the platelets were counted and their number adjusted to between $1 \cdot 10^9$ and $2 \cdot 10^9$/ml. The pH of the platelet suspension was adjusted to 7.2 by addition of Tris base. For some experiments we included 1.5 μM aprotinin. 100 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride. Stimulation of the platelets was provided by addition of bovine thrombin 0.1 U/ml and 0.5 U/ml, ADP 5 μM, 10 μM and Ca^{2+}-ionophore A23187, 2–5 μM. After two gentle inversions, the platelet suspension was allowed to remain at room temperature for 5 min without agitation. Platelet shape change was verified by phase microscopy. Platelet suspensions were then subjected to five strokes with the teflon coated pestle of a glass tissue grinder (clearance 0.056 mm). The homogenized platelet suspension was then centrifuged at $950 \times g$ for 25 min. To concentrate the pseudopodia, the supernatant was removed and centrifuged at $2300 \times g$ for 25 min. The platelet cell bodies, obtained as a pellet of the first centrifugation as well as the pseudopodia of the second centrifugation were resuspended in a small volume of Mes buffer. Up to this point all procedures were performed at room temperature.

Purification of platelet pseudopodia

For these experiments the pseudopodia containing the supernatant of the cell homogenate was centrifuged at $5000 \times g$ for 45 min at 4° C. The resultant pellet was resuspended in Mes buffer and layered on top of an interrupted sucrose gradient ranging from 40% to 5% in 5% increments. The sucrose gradients were centrifuged at $28000 \times g$ for 30 min at 4° C and individual fractions collected by puncturing the bottom of the tube. Optical absorbance and protein content were measured on each 1 ml fraction.

Protein was assayed according to Lowry et al. [17] using bovine serum albumin as standard.

Lipid analyses

Washed suspensions of intact whole platelets, cell bodies or platelet pseudopodia were extracted with chloroform/methanol (1 : 2, v/v) containing butylated hydroxytoluene (BHT), 50 μg/ml. To 3 ml of cell extract were added 0.3 ml of 0.2 M EDTA, 1 drop 88% formic acid and 11.2 ml of chloroform/methanol. An additional 3.75 ml of 2 M KCl and 3.75 ml of chloroform with BHT was added. The lipid extract was recovered and the remainder was reextracted with 7.5 ml chloroform containing BHT. The combined extracts were concentrated under N_2 and redissolved in 0.2 ml chloroform/methanol. Of this, one portion was used for phospholipid analysis, the other to prepare fatty acid methyl esters for gas chromatography.

Fatty acids were transesterified with boron trifluoride in 14% methanol. To each lipid extract was added as internal standard 5 μg heptadecanoic acid. Transesterification was carried out at 100° C for 90 min under N_2. The fatty acid methyl esters were extracted by adding 2 ml of ultra pure water and 3 ml petroleum ether. The ether layer was transferred to a screw top tube and evaporated under N_2 while the original solution was extracted with another 3 ml of petroleum ether. The two extracts were combined, evaporated and dissolved in 25–50 μl methylene chloride containing BHT (5 mg/ml).

Gas chromatography was performed in a model 8500 Perkin-Elmer gas chromatograph using a capillary column SP-2230 (Supelco). The injector had a 1/10 split ratio. Bleed calibration of a blank run was automatically subtracted from the experimental run. After an initial isothermal period of 5 min at 85° C, the temperature was raised to 185° C at 20° C/min. It was kept at this temperature for 20 min and then raised to 200° C again at 20° C/min and remained at the final temperature for 6 min. This temperature program allowed elution of all the fatty acid methyl esters within a 37 min period. When gas chromatographic analyses could not be performed immediately, the extracted fatty acid methyl esters were stored at −80° C. Most of the fatty acid methyl esters were identified by comparing their retention times with those of authentic standards. Certain fatty acid methyl esters were identified by mass spectroscopic analyses which were performed on a Finnegan mass spectrometer.

Platelet phospholipids were analyzed by thin layer chromatography as previously described [18]. Lipids were visualized either by exposure to iodine vapor or by spraying the TLC plates with Rhodamine B and then exposing them to fluorescent light. Lipid phosphorus was measured according to Chen et al. [19]. A standard curve was prepared using sphingomyelin.

Extraction and measurement of alpha-tocopherol

Platelet membranes or cell bodies suspended in 0.05 M Tris-HCl and 0.14 M NaCl, pH 7.2 (TBS) were extracted with 3 vol. of *n*-hexane [20]. An aliquot of the suspension of each cell fraction was set aside for the determination of protein concentration. After vigorous shaking for 2 min the mixture was allowed to settle. The organic top layer was removed and dried under N_2. The dry residue was taken up into 1 vol. of methanol and after spin filtration through a 0.2 μm filter (Millipore Corp., Bedford, MA) the extract was again evaporated under N_2 and dissolved in an appropriate volume of high pressure liquid chromatography grade methanol. This solution was analyzed on an Ultrasphere ODS 5-μm column (4.6 × 250 mm) (Rainin Instr. Co., Woburn, MA). This column was developed with methanol/water (95 : 5, v/v) at a flow rate of 1 ml/min. Optical absorbance measurements were taken at 230 and 290 nm. Alpha-tocopherol usually had a retention time of 16.4–17.2 min. The individual chromatograms were analyzed and quantified by an IBM 9000 computer using a chromatographic application program (CAP3, IBM). A standard curve of alpha-D-tocopherol was prepared, ranging from 50 ng to 10 μg.

Electrophoretic separation of platelet proteins

High-resolution SDS-polyacrylamide gradient gel electrophoresis was performed as previously described [21,22]. Molecular weights were estimated by comparison with a series of high and low molecular weight standards that were included with each electrophoretic separation. Proteins were identified by silver stain. Electrophoretic blotting onto nitrocellulose or Immobilon-PVDF sheets was performed according to Towbin et al. [23]. A series of prestained standards was included in order to have a molecular weight yardstick and also to be able to determine the completeness of the transfer.

Two-dimensional electrophoresis was performed essentially according to O'Farrell [24].

Labeling of membrane proteins

Glycoprotein labeling. Exofacial glycoproteins of platelets were subjected to metaperiodate oxidation of terminal sialic acid residues followed by reduction in the presence of biotinhydrazide. The platelets were stimulated first with thrombin and pseudopodia and platelet cell bodies separated as described above. After washing pseudopodia and cell body preparations repeatedly with 0.05 mol/l phosphate buffer containing 0.14 M NaCl, pH 7.4 (PBS), they were resuspended in PBS and incubated with 10 mM biotinhydrazide and 1 mM $MnCl_2$ for 30 min at room temperature. The cells were then separated by centrifugation, washed × 3 with PBS and finally solubilized in 0.0625 M Tris, 1 mM Na_2EDTA and 1% SDS at a concentration of approx. 1 mg protein/ml.

The presence of biotin in the separated platelet glycoproteins was shown by reacting the Western blots prepared on Immobilon-PVDF with gold-conjugated streptavidin (diluted 1 : 100 with 0.1% bovine serum albumin (BSA) dissolved in PBS and containing gelatin (1 : 20, v/v)). The procedure of treating electrophoretic transblots was otherwise as described before [22].

[^{32}P]ATP labeling of platelets. Cell bodies and pseudopodia of platelets were prepared as described above. Each of the two platelet components were then suspended in an incubation buffer consisting of 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 0.5 mM EGTA, 5 μM cAMP, 100 mg% glucose and 0.3% BSA (pH 7.4). After temperature equilibration to 30 °C to each incubation mixture was added to 40 μCi [γ-^{32}P]ATP. The suspensions were incubated for 5 min at 30 °C. The reactions were terminated by addition of trichloroacetic acid (TCA) to a final concentration of 5%. After allowing the samples to remain at 4 °C for 10 min, the precipitates were sedimented by centrifugation following which the precipitates were redissolved in 1 M NaOH. After complete solubilization, the samples were reprecipitated with TCA. This procedure was repeated twice and finally the precipitate was neutralized and dissolved in SDS-solubilizing buffer for subsequent SDS-polyacrylamide gradient gel electrophoresis or in lysis buffer for two-dimensional electrophoresis according to O'Farrell [24].

Electron microscopy

Intact platelets, their cell bodies and platelet pseudopodia were pelleted and fixed with Karnovsky's solution for 2 h followed by three 10-min rinses with cacodylate buffer. The samples were postfixed with 0.1% osmium tetroxide in cacodylate buffer for 1 h. After a graded series of dehydration steps with increasing concentrations of ethanol, the samples were embedded in Quickmix epoxy. Thin sections (600–700 Å) were stained with uranyl acetate and lead citrate and were then examined with a Hitachi HS-9 electron microscope.

Materials

Bovine thrombin was obtained from Parke-Davis, Morris Plains, NJ and ADP from Sigma Chemicals, Inc., St. Louis, MO. Fatty acid methyl esters were obtained either from Supelco, Bellefonte, PA or NuChek Prep, Elysion, MN. Boron trifluoride in 14% methanol was used in form of a prepared solution obtained from Analabs, Norwalk, CT. Alpha-D-tocopherol, research grade was purchased from Serva Biochemicals, NY. Antibody to PI^{A1} was a generous gift of Dr. R. Aster, Milwaukee, WI. Biotinhydrazide was a product of Calbiochem, San Diego, CA. Gold-conjugated streptavidin (AuroProbe BL plus streptavidin) was obtained from Janssen Life Sciences Products, Piscataway, NJ. All other chemicals were of highest purity available.

Results

In our initial experiments we established the optimal method for separating pseudopodia from platelet cell bodies. Comparison of motor driven versus hand operated homogenization, both for a total of 5 up and down strokes, showed a slightly higher yield of pseudopodia by the latter. Using ADP (10 μM) as platelet stimulant, it yielded 4.2% of the total platelet protein in pseudopodia whereas the motor driven homogenization method yielded 3.3% in pseudopodia. A comparison of different homogenizer clearances, the space between the Teflon coated pestle and the glass wall of the homogenizer, was also performed. We tested clearances that ranged from 30 μm to 100 μm. A 56 μm clearance appeared to be the optimal one, but differences between them were very small and statistically insignificant (Table I). The combined recovery of pseudopodia and cell bodies compared to the amount of protein of the intact platelets varied between 82 and 94%.

Destruction of platelets by the shear force generated by the homogenization procedure was analyzed by measuring lactate dehydrogenase (LDH) activity released into the medium. There was evidence of some destruction, however the total enzyme activity released was relatively small compared to the amount of lactate dehydrogenase present in intact platelets (Table I). The total LDH activity was found to be 4.4 ± 0.3 U/1 · 10^9 platelets. ADP-stimulated pseudopodia formation caused less release of LDH than that induced by thrombin (hand operated homogenization, 3.7% ± 0.4, motor driven homogenization 5.1% ± 0.6). Release from platelet granules was also measured by preloading platelets with radioactive serotonin and then subjecting them to the homogenization procedure. The results in Table II show a modest amount of serotonin release from the homogenized platelets. Light microscopic examination of the platelets showed little destruction due to homogenization. This could be more clearly demonstrated by ultrastructural examination of platelets subjected to these homogenization procedures. The remarkable preservation of the electron dense granules and other subcellular organelles is apparent in the electron micrographs shown in Fig. 1.

Other experiments were performed to determine the optimal method for separating platelet pseudopodia in pure form as well as preparing platelet cell bodies free of contaminating pseudopodia. Using an interrupted sucrose gradient we were able to obtain 'pure' preparations of platelet pseudopodia that appear to be uncontaminated by cell bodies as demonstrated by electron microscopy (Fig. 1). Although the preparation of ultra pure pseudopodia and cell bodies sacrificed a considerable amount in the yield of these respective components, especially the former, for certain experiments we relied on this methodology in order to obtain pure uncontaminated preparations of these respective cell parts.

The analysis of phospholipids revealed major differences between cell bodies and pseudopodia (Table III). There was a considerable incrase in lysophospho-

TABLE I

Effect of pestle clearance on recovery of platelet pseudopodia [a] *and LDH release* [b]

Clearance (μm)	Pseudopodia (% of total platelet proteins)	Release of LDH (% of total platelet LDH) [c]
30	13.3 ± 1.2	14.8 ± 2.0
56	14.1 ± 1.2	13.9 ± 1.5
100	15.7 ± 1.6	15.8 ± 3.0

[a] Pseudopodia formation was induced with thrombin 0.1 U/ml.
[b] Hand operated homogenization (5 strokes) of platelet suspension.
[c] LDH release was measured as previously described [34].

TABLE II

Release of serotonin during formation of platelet pseudopodia

Stimulant	Concentration	5-Hydroxy-[^{14}C]tryptamine (% release) [a,b]
ADP	5 μM	19.2 ± 2.4
ADP	10 μM	22.8 ± 3.1
Thrombin	0.1 U/ml	32.1 ± 4.3
Ca^{2+} ionophore	2 μM	27.8 ± 3.8
Thrombin (without homogenization)	0.1 U/ml	14.3 ± 2.9

[a] Mean ± 1 S.D. of four experiments.
[b] Serotonin release was measured as previously described [35].

TABLE III

Phospholipid distribution in platelet pseudopodia and cell bodies

Phospholipid	Cell bodies	Pseudopodia
	μg/P_i 1 · 10^{10} platelets [b]	
SM	6.63 ± 1.05	1.48 ± 0.20
PC	32.86 ± 2.10	3.33 ± 0.29
PS	12.97 ± 1.42	1.57 ± 0.22
PI	10.25 ± 1.03	1.40 ± 0.21
PE	22.52 ± 1.72	3.24 ± 0.28
LPC	5.56 ± 0.85	1.14 ± 0.19
	Percentage distribution	
SM	6.8 ± 0.4 [b]	11.4 ± 0.6
PC	33.7 ± 1.3 [b]	25.6 ± 1.1
PS	13.3 ± 0.9	12.1 ± 0.7
PI	10.5 ± 0.6	10.8 ± 0.7
PE	23.1 ± 1.2	24.9 ± 1.1
LPC	5.7 ± 0.4 [b]	8.8 ± 0.6

[a] Mean ± 1 S.D. of six experiments.
[b] Statistically significant difference between pseudopodia and platelet cell bodies; $P < 0.0005$.

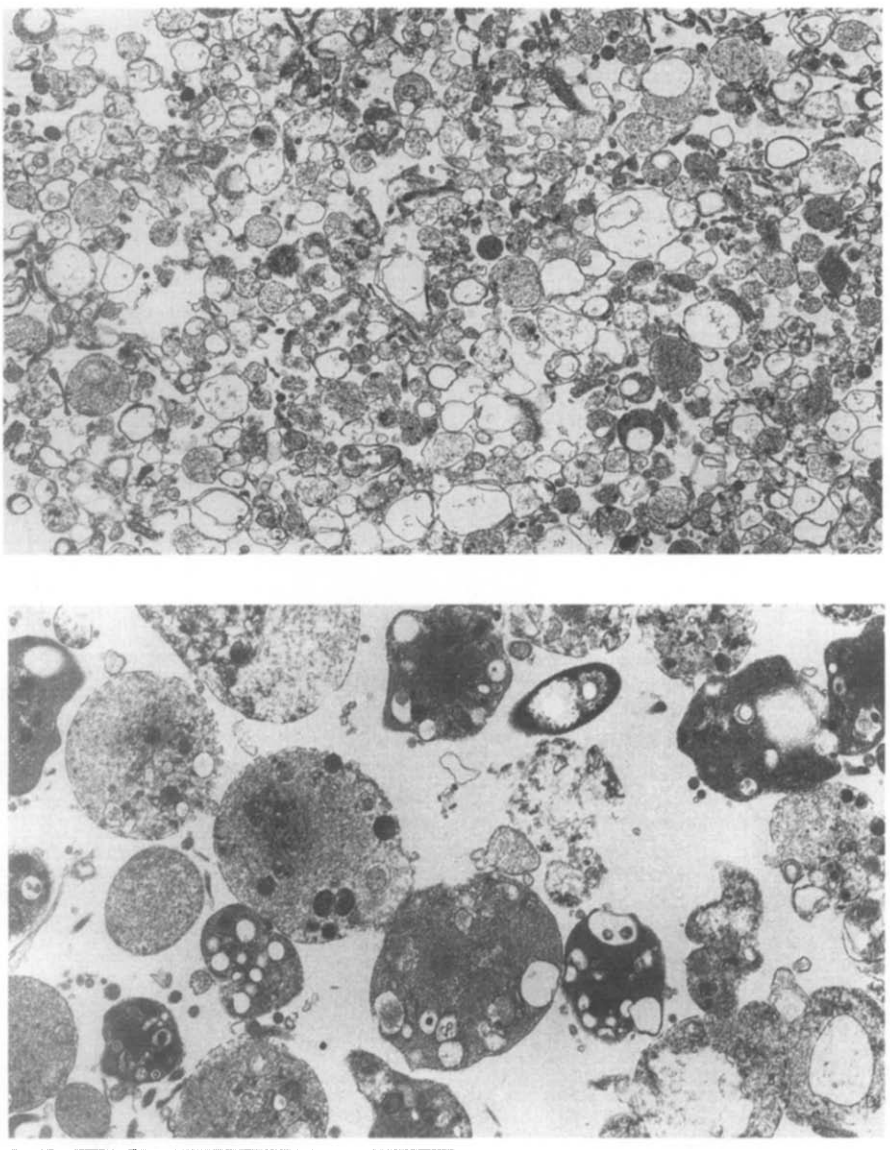

Fig. 1. Electron micrographs of pseudopodia (A) and platelet cell bodies (B). Original magnification, ×16500; as shown, ×14025.

lipids in the pseudopodia fraction as well as an increase in sphingomyelin. On the other hand, phosphatidylcholine and phosphatidylethanolamine showed modest decreases in pseudopodia compared to cell bodies. Major differences existed also in the fatty acids in these two fractions (Table IV). The total fatty acids of pseudopodia differed from their counterparts in cell bodies by showing a large increase in mono-, di- and tri-ene fatty acids. Polyunsaturated fatty acids, as well as saturated fatty acids, were slightly decreased in the pseudopodia fraction as compared to the cell bodies. There was a major increase of 22:1 and 18:1 in pseudopodia, whereas significant decreases occurred in $20:4(n-6)$. These results did not depend upon the stimulant used to induce platelet shape change prior to homogenization.

Quantitative evaluation of pseudopodia formation by various stimulants is presented in Table V. Thrombin was by far the most effective agonist, producing pseudopodia in a predictable manner. Their protein content varied between 17 and 20% of that of the remaining platelet cell bodies. ADP, on the other hand, was the stimulant causing the least abundant pseudopodia formation. It ranged between 5 and 8% of cell bodies. The other agonists, as shown in the table, fall between these two extremes. Measurements of lipid extractable phosphorus in pseudopodia and platelet cell bodies are in general agreement with the protein assays.

The distribution of alpha-tocopherol in platelets obtained from individuals on supplemental vitamin E is shown in Table VI. There was a notable discrepancy in the distribution between platelet cell bodies and pseudopodia. Extraction of alpha-tocopherol from membrane portions of the two respective platelet fractions clearly demonstrated a significantly higher specific ac-

TABLE IV

Profile of total fatty acids in platelet pseudopodia and cell bodies

Fatty acid	% Distribution [a]	
	Pseudopodia	Cell bodies
16:0	10.5±0.8	11.1±0.8
16:1	0.6±0.15	0.2±0.04
18:0	13.8±0.9	15.9±0.9
18:1	8.5±0.8[b]	4.2±0.6
18:2	7.6±0.9	6.6±0.8
18:3	2.3±0.4[b]	0.3±0.05
20:0	2.2±0.4	1.4±0.1
20:1	3.5±0.6	4.6±0.7
20:3	2.2±0.5	1.2±0.3
20:4	18.5±1.2[b]	26.9±1.1
22:0	0.8±0.2	0.6±0.15
22:1	17.6±1.4[b]	6.2±0.8
20:5	0.1±0.02	0.2±0.03
24:0	1.5±0.2	1.7±0.12
24:1	4.5±0.6	4.3±0.5
22:6	0.5±0.2	2.2±0.4

[a] Mean±1 S.D. of six experiments.
[b] Statistically significant difference between pseudopodia and platelet cell bodies; $P < 0.0005$.

TABLE V

Pseudopodia formation induced by different platelet agonists

Agonist	Concentration	Pseudopodia recovery (% of total platelet protein) [a]
Thrombin	0.1 U/ml	13.1±1.3
Thrombin	0.5 U/ml	19.2±2.8
ADP	5 μM	2.4±0.3
ADP	10 μM	4.9±0.4
Ca^{2+} ionophore	2 μM	7.6±0.8

[a] Mean±1 S.D. of four experiments.

tivity of alpha-tocopherol in membranes of platelet cell bodies than in those drived from pseudopodia. Although the determination of alpha-tocopherol in platelet pseudopodia yielded values at the extreme lower end of the standard curve of alpha-tocopherol, the results

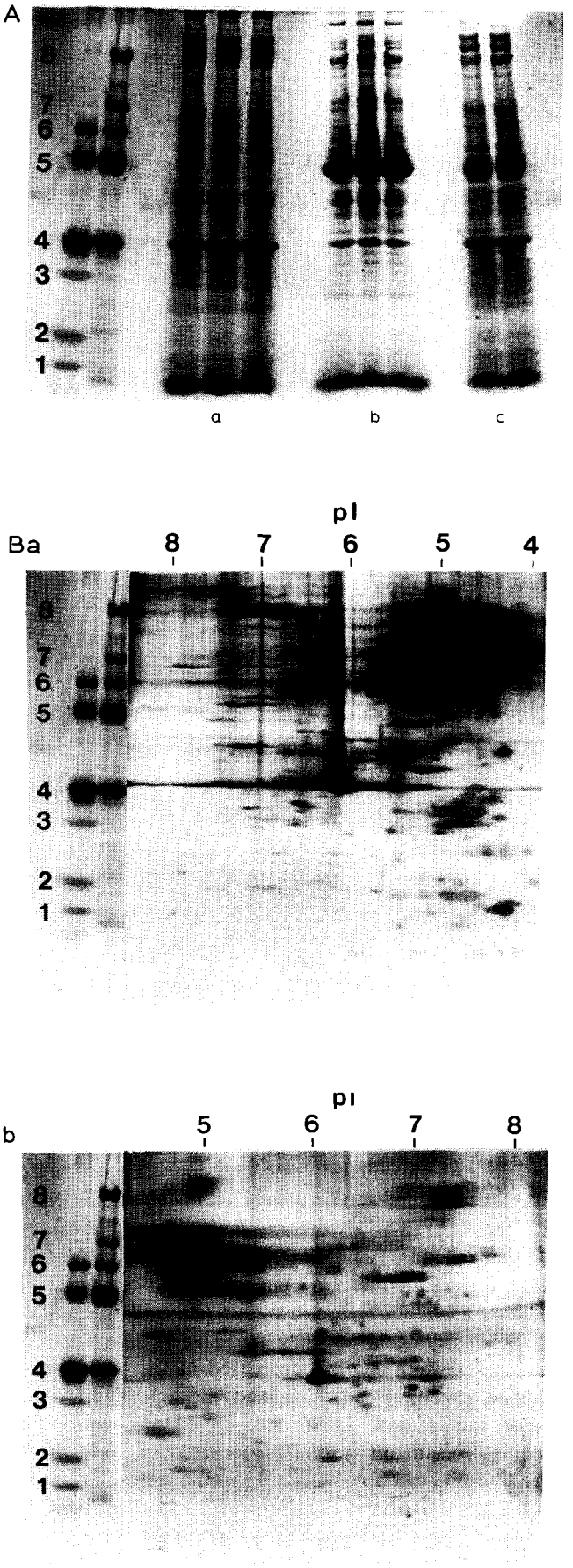

Fig. 2. (A) Evaluation of platelet proteins derived from platelet bodies (a), platelet pseudopodia (b), and whole platelets (c) by SDS-polyacrylamide gradient gell electrophoresis. High and low molecular weight standards are shown on the left side of the figure. Platelet pseudopodia were prepared by stimulating platelets with thrombin, 1 U/ml. ADP, 10 μM and Ca^{2+} ionophore A23187, 5 μM. Molecular mass standards: 1, 14.4 kDa; 2, 21.5 kDa; 3, 31 kDa; 4, 45 kDa; 5, 66.2 kDa; 6, 97.4 kDa; 7, 116 kDa; 8, 200 kDa. 100 μg of protein was applied to each lane. (B) Evaluation of proteins derived from platelet cell bodies (a) and pseudopodia (b) by two-dimensional electrophoresis [24]. The position of molecular mass standards is indicated on the left side of the figure and pH values are shown along the top of the figure. The numbers correspond to those given in legend to Fig. 2A.

TABLE VI

Distribution of alpha-tocopherol between platelet pseudopodia and cell bodies

Platelet fraction	Alpha-tocopherol content [a]	
	μg/mg protein	ng/μg P_i
Cell bodies		
Membranes	1.48 ± 0.1	21.3 ± 0.15
Lysate	0.13 ± 0.01	–
Pseudopodia		
Membranes	0.71 ± 0.05	10.4 ± 0.1
Lysate	0.03 ± 0.01	–

[a] Mean ± 1 S.D. of three experiments.

nevertheless were reproducible as shown by the relatively small standard deviations obtained.

Investigation of the protein distribution in platelet pseudopodia and cell bodies by one- and two-dimensional electrophoresis on high-resolution SDS-polyacrylamide gradient gels showed that platelet pseudopodia are rich in a protein of approximate 68 kDa (Fig. 2). The protein apparently has few terminal sialic acid residues as metaperiodate oxidation followed by reduction with biotinhydrazide yielded only a faint band in that area upon reaction with gold-conjugated streptavidin (Fig. 3). On the other hand, this protein reacted strongly when a Western blot was treated with Pl^{A1}

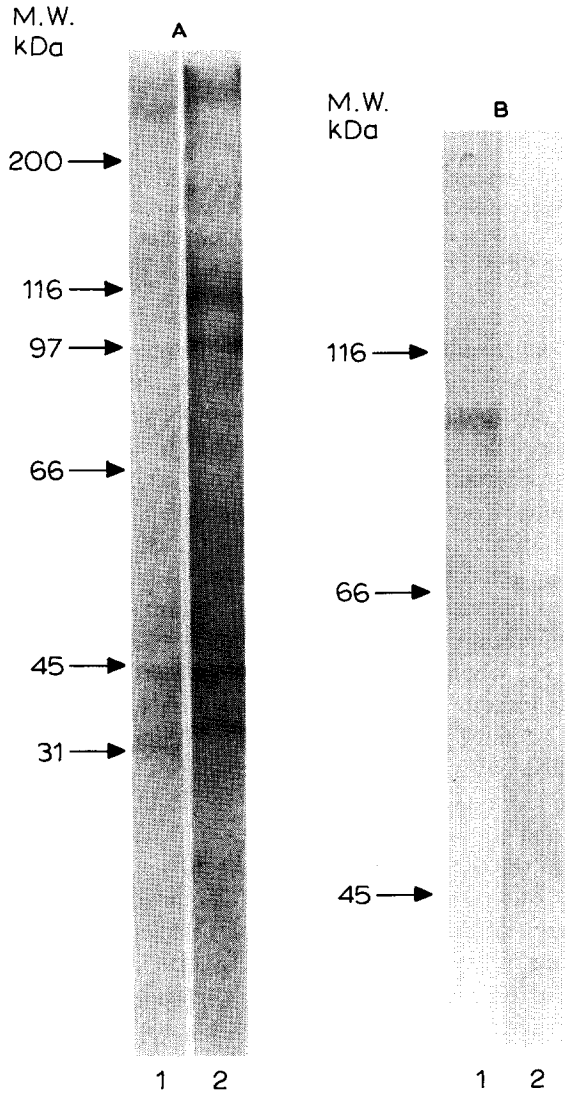

Fig. 3. Identification of sialic acid containing glycoproteins (A) and Pl^{A1} reactive proteins (B) in platelet pseudopodia (1) and cell bodies (2). SDS-PAGE resolved platelet proteins were prepared as described under Methods (the gel shown in (B) was not reduced with dithiothreitol), blotted onto Immobilon PVDF membranes which were then treated with gold conjugated streptavidin or with antiserum to Pl^{A1} and gold-conjugated anti-human IgG. Subsequent silver enhancement was used to demonstrate the position of the reactive bands. Molecular mass standards identified by number are identical to those in legend to Fig. 2A.

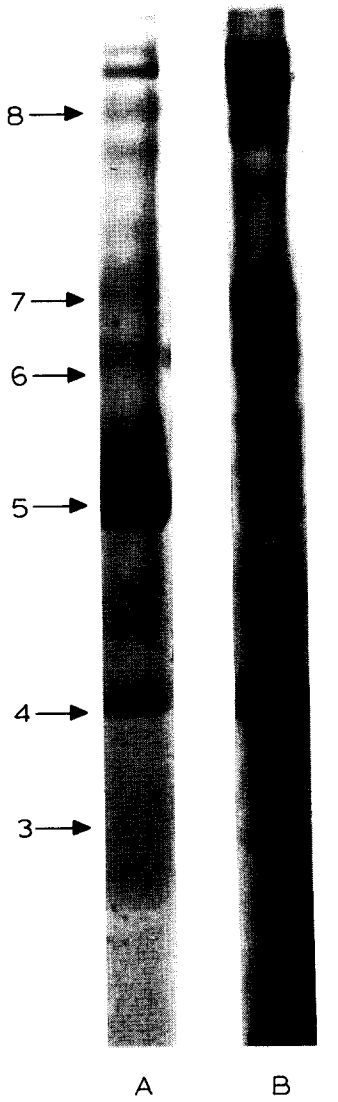

Fig. 4. Analysis of platelet pseudopodia by SDS-polyacrylamide gradient gel electrophoresis. Platelets were stimulated with Ca^{2+} ionophore A23187, 5 μM. After 1 min calpain inhibitor II, 0.5 mM (B) or an equal volume of dimethylsulfoxide (solvent for calpain inhibitor II) (A) was added and incubation continued for 5 min at room temperature. Pseudopodia were then isolated as described under Methods and Materials. Molecular mass standards are indicated on the left by the same numbers as given in legend to Fig. 2A.

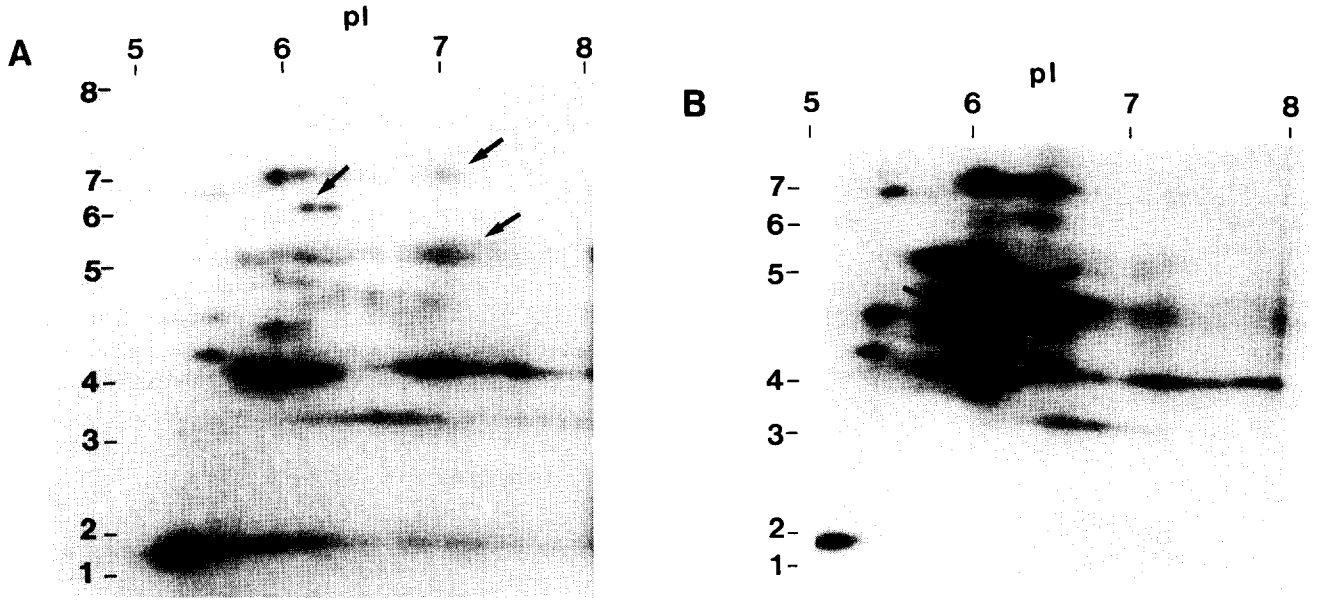

Fig. 5. Autoradiograms of ^{32}P-labeled platelet proteins derived from pseudopodia and cell bodies. Platelet pseudopodia (A) and cell bodies (B) were labeled with [γ-^{32}P]ATP, the proteins were separated by two-dimensional electrophoresis, electrophoretically transblotted onto Immobilon PVDF and autoradiographs were prepared. Molecular mass standards are identical to those shown in Fig. 2A. The arrows indicate radioactive peptides that differ between pseudopodia and platelet cell bodies.

antibody. Although various protease inhibitors added to the platelet suspension before the preparation of pseudopodia had no significant effect on the appearance of the SDS gradient gels, the addition of calpain inhibitor II (*N*-acetylleucylleucylnormethioninal; final concentration (0.5 mM) 1 min before or after activation of platelets, but before preparation of pseudopodia produced a striking reduction of the 68 kDa polypeptide (Fig. 4).

The phosphorylation of platelet proteins with [γ-^{32}P]ATP revealed a number of differences between pseudopodia and platelet cell bodies (Figs. 5A and B). Some of them were only quantitative in nature, but there were at least three changes (112 kDa, 66–70 kDa and 95 kDa) in which the radioactive peptides visible in pseudopodia were absent from cell bodies. Two of the labeled peptides were absent from pseudopodia and were only present in cell bodies (55 kDa and 42 kDa).

Discussion

In this study, we have used a unique property of platelets in an attempt to prove that lipids in the plasma membrane are not homogenously distributed. The remarkable shape change which occurs upon stimulation of platelets afforded us the opportunity to separate specific areas of the outer plasma membrane of platelets and examine their morphological and biochemical characteristics. The technique we have employed to separate pseudopodia is relatively simple and rapid, but does require a certain amount of time during which redistribution of lipids could occur. Nevertheless, we believe that our results show substantial differences between the two separated cell components. A possible realignment of lipids as well as proteins occurring after the platelets have undergone shape change would have to be considered of relatively minor importance. It should be pointed out that the preparation of platelet pseudopodia differs significantly from that of the so-called platelet microvesicles [11–14]. The latter, if separated from platelets, remain in the supernatant of a high-spin centrifugation (6000 rpm $\times$ 15 min Sorvall; HG-4L rotor) [12] whereas our material is pelleted at relatively low *g* forces (2300 $\times$ *g*). We cannot exclude that there may be some contamination of our pseudopodia preparation with such microparticles, but it is not the major source of our material. In addition, we have included in many of our studies a further separation step in the form of a sucrose gradient.

As our results show, there are some significant differences in the lipid structure of platelet pseudopodia and the remaining cell bodies. Lysophospholipids appear to be concentrated in the pseudopodial fraction which would suggest that phospholipase A_2 predominates in these structure or, at least, is one of the primary sites where it is activated. The other significant changes in phospholipids were the differences in sphingomyelin and phosphatidylcholine between pseudopodia and cell bodies. Since sphingomyelin is known to be rich in saturated fatty acids [25], the increase observed in the pseudopodial fraction does not fit the picture of a structure otherwise rich in unsaturated fatty acids.

This change may be offset, however, by a reduction in phosphatidylcholine, the primary source of saturated

fatty acids in platelet membranes. Measurements of the fatty acid distribution in the two respective cell components showed that the ratio of saturated to unsaturated fatty acids is clearly in favor of the latter in pseudopodia compared to cell bodies, especially when mono- and diene fatty acids are compared. Physical/chemical analyses of the configuration of mono-, di- and polyunsaturated fatty acids have clearly shown that maximal deviation from linearity manifesting itself in greatest shortening, occurs with triene fatty acids. Fatty acid residues of higher unsaturation assume a more linear configuration and thus cause less of a perturbation of the lipid bilayer than mono- and di-unsaturated fatty acids [9]. We speculate that such areas of greater concentration of fatty acids of lower degrees of unsaturation may facilitate the formation of pseudopodia upon platelet activation. We speculate that the areas where they occur exert less resistance to the projection of cell contents than do areas where the lipid layer is less perturbed. These membrane regions can be assumed to display greater packing of fatty acid residues in the plane of the lipid matrix and exert greater resistance toward the extrusion of cell contents.

Conclusions about platelet membrane heterogeneity are predicated on the ability to directly compare plasma membranes of pseudopodia with those of platelet cell bodies. As the latter contain many if not most of the platelet granules, differences in lipid composition could originate from the presence of subcellular organelles. The careful and detailed study by Marcus et al. [25] has clearly shown that there is no significant difference in phospholipids and fatty acid distribution between platelet membranes and whole platelets (containing platelet granules). Only triacylglycerols and free fatty acids exhibited a minor difference in the distribution of plamitic and linoleic acid. However, the relative contribution of these two neutral lipid fractions, 0.48 and 0.44%, respectively, of total platelet lipids, is so small that one can safely assume that there are no differences when comparing total fatty acid distribution between whole platelet cell bodies and platelet membranes.

Our studies also show that inhomogeneities in the plasma membrane are not restricted to the lipid moieties, but are also observed among proteins. The predominance of a protein of approximately 68 kDa in the pseudopodial fraction gave a most obvious demonstration of this point. We believe that this protein is similar to the one described by Rotman et al. [26] although attempts to identify this peptide as a glycoprotein have been inconclusive. Using metaperiodate oxidation of sialic acid residues followed by reduction in the presence of biotinhydrazide, we were able to obtain a very faint reaction with gold-conjugated streptavidin. Our attempts at identification of the nature of this protein, however, were successful. Kornecki et al. [27] have identified a Pl^{A1} alloantigen domain on a 66 kDa protein derived from glycoprotein IIIa of human platelets treated with thrombin or chymotrypsin. The strong reaction we obtained when Western blots containing thrombin-induced platelet pseudopodial proteins were treated with a potent antiserum to Pl^{A1} proved that this protein contained an epitope for this antibody and was very persuasive evidence that it probably was produced by a prtoeolytic process from gp IIIa. As we found similar changes after preparing pseudopodia with ADP and Ca^{2+} ionophore A23187, we postulate that an activation of proteolytic enzymes in the platelet is either associated with the agonist induced shape change, or occurs as a consequence of the homogenization procedure that was used to prepare pseudopodia. In fact, the addition of calpain inhibitor II before platelet activation or after stimulation with an agonist, but before preparation of pseudopodia strongly inhibited the appearance of this polypeptide as shown by SDS-polyacrylamide gradient gel electrophoresis (Fig. 4). This suggests that the production of the 68 kDa protein is the result of specific proteolytic action. The reason why calpain affects primarily a protein in the platelet pseudopodia is not clear. The important aspect, however, is the apparent inhomogeneity of this effect in the two cell components.

Support for an inhomogeneous distribution of platelet proteins was also obtained by labeling pseudopodia and platelet cell bodies with [γ-^{32}P]ATP. We were able to observe subtle but reproducible changes between these two platelet components. Whether there is any functional significance attached to these changes remains to be determined.

The question arises whether the changes observed in proteins between pseudopodia and platelet cell bodies reflect true heterogeneity of the platelet plasma membrane. We believe that the answer is affirmative as our attempts to characterize such changes on one hand utilized methodology that labels only plasma membrane proteins ([γ-^{32}P]ATP and metaperiodate oxidation of sialic acid residues) and on the other were productive only in pseudopodia.

Alpha-tocopherol, an agent capable of inhibiting platelet adhesion [15,28] was shown to distribute itself unequally among pseudopodia and platelet bodies. Enrichment of platelet membranes with alpha-tocopherol can change membrane fluidity, decreasing it at temperatures below 23°C and raising it above the unsupplemented controls at temperatures greater than 25°C [29]. The results that we obtained in these experiments in which pseudopodia formation was induced at room temperature are thus in agreement with our previous finding.

The alpha-tocopherol induced inhibition of platelet pseudopodia formation appears to be unique for an 'anti-platelet' agent such as alpha-tocopherol [15]. Platelet shape change may be inhibited by agents that

raise cyclic AMP in platelets, e.g., prostacyclin and other cyclic AMP stimulating prostaglandins [30] but has never been described for a substance which does not cause a large increase in the cyclic AMP level of the cell. In previous studies we have shown that alpha-tocopherol can inhibit cyclic nucleotide phosphodiesterase but it is uncertain whether cyclic AMP levels, which we did not measure, are influenced in platelets [31]. The inhibitory effect of alpha-tocopherol on platelet pseudopodia formation appears to be maximal at or about a daily intake of 400 IU [15]. The mechanism of action by which alpha-tocopherol can inhibit pseudopodia formation has not yet been elucidated.

Infante [32] proposed that alpha-tocopherol can enhance the activity of fatty acid desaturases and elongases in cells. This hypothesis is based in part on experimental evidence obtained in vitro, measuring the effect of alpha-tocopherol supplementation of culture media on the fatty acid composition of mouse fibroblasts [33]. Whether alpha-tocopherol supplementation of the diet has a similar effect on human platelets remains to be shown, but if such a process occurs, especially in discrete areas of the membrane, it could change the formation of pseudopodia upon platelet stimulation.

We feel that our studies have provided good evidence for the existence of discrete areas of heterogeneity in platelet membranes. Their functional significance appears to be quite obvious in the case of platelets where such unique membrane domains appear to have a distinct relation to platelet activation.

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