Production of Phosphatidylinositol 3,4,5-Trisphosphate and Phosphatidic Acid in Platelet Rafts: Evidence for a Critical Role of Cholesterol-Enriched Domains in Human Platelet Activation†

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Received May 7, 2001; Revised Manuscript Received August 9, 2001

ABSTRACT: Glycosphingolipid- and cholesterol-enriched membrane microdomains, called rafts, can be isolated from several mammalian cells, including platelets. These microdomains appear to play a critical role in signal transduction in several hematopoietic cells, but their function in blood platelets remains unknown. Herein, we first characterized the lipid composition, including the fatty acid composition of phospholipids, of human platelet rafts. Then their role in platelet activation process was investigated. Interestingly, thrombin stimulation led to morphological changes of rafts correlating with the production of lipid second messengers in these microdomains. Indeed, we could demonstrate for the first time that a large part of the stimulation-dependent production of phosphatidic acid and phosphoinositide 3-kinase products was concentrated in rafts. Moreover, cholesterol depletion with methyl-β-cyclodextrin disrupted platelet rafts, dramatically decreased the agonist-dependent production of these lipid signaling molecules, and impaired platelet secretion and aggregation. Cholesterol repletion restored the physiological platelet responses. Altogether our data indicate that rafts are highly dynamic platelet membrane structures involved in critical signaling mechanisms linked to the production of lipid second messengers. The demonstration of phosphatidylinositol 3,4,5-trisphosphate production in rafts may have general implications for the understanding of the role of this key second messenger found ubiquitously in higher eucaryotic cells.

Separate functional domains with different lipid compositions can coexist in biological membranes (*1*). Glycosphingolipid- and cholesterol-enriched domains called rafts, DIGs[1] (detergent-insoluble glycosphingolipid-enriched domains) or DRMs (detergent-resistant membranes) have been isolated from a number of cell types (*2*, *3*). These membrane microdomains, consisting of tightly packed lipids, are in liquid-ordered phase and display lateral mobility (*4*, *5*). In cells expressing caveolin, rafts can coexist with a subset of lipid microdomains found in cell surface invaginations called caveolae (*6*, *7*). Cholesterol is known to play an important role in the lipid phase organization and has a potent effect on raft or caveolae stabilization (*4*). Alterations in the cholesterol/sphingolipid/phospholipid ratio may modify the structure of these microdomains and thereby their functionality. Rafts have been shown to play a critical role in cell signaling, especially in hematopoietic cells (*3*, *8*, *9*). Recently, their participation in the membrane reorganization required for antigen receptor signaling in T cells (TCR) has been demonstrated. Indeed, TCR-mediated signal transduction cascade appears to be dependent on the recruitment of signaling molecules within rafts (*10*, *11*). Perturbation of the structure of these microdomains dramatically reduces TCR-mediated cell stimulation (*10*, *11*). Thus, rafts are considered as membrane centers where physical association of functional partners occurs to trigger lymphocyte activation.

Rafts can also be isolated from blood platelets (*12*) on the basis of their low density and their resistance to solubility by nonionic detergent (*13*). Rafts isolated from human platelets lack caveolin, are enriched in CD36, and contain tyrosine kinases of the Src family (*12*, *14*), suggesting that they may be involved in platelet signal transduction. How-

† This work was supported by grants from European Union Biomed 2 Program BMH4-CT-97 2609, Association pour la Recherche contre le Cancer, and La Ligue Nationale contre le Cancer.

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[1] Abbreviations: BSA, bovine serum albumin; DAG, diacylglycerol; DIGs, detergent-insoluble glycosphingolipid-enriched domains; DRMs, detergent resistant membranes; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; $InsP_3$, inositol trisphosphate; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T cells; MβCD, methyl-β-cyclodextrin; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; SM, sphingomyelin; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PH domain, pleckstrin homology domain; PI, phosphoinositides; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PS, phosphatidylserine; PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol monophosphate; $PtdInsP_2$, phosphatidylinositol bisphosphate; PtdOH, phosphatidic acid; SHIP, SH2 domain containing inositol 5-phosphatase; TCR, T-cell receptor.

10.1021/bi0109313 CCC: $20.00
Published on Web 11/20/2001

ever, the role of these cholesterol-enriched domains in platelet functions is still unknown. Interestingly, a correlation between cholesterol content and platelet responsiveness has been observed about 20 years ago (*15*−*17*). The molecular basis of this mechanism is still unknown but it is tempting to speculate on the role of cholesterol-enriched microdomains.

Phosphoinositides and phosphoinositide-derived second messengers are involved in a wide diversity of signaling pathways (*18*). The hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] by phospholipase C (PLC) represents a key signaling event through the generation of diacylglycerol (DAG) and inositol trisphosphate ($InsP_3$), involved in the activation of protein kinase C (PKC) and in the increase of cytosolic calcium level, respectively. Recent studies showed that rafts and caveolae appear to be preferential membranous sites for the turnover of PtdIns(4,5)P_2 (*19*−*21*). D-3 phosphoinositides such as PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 also play a critical role in the spatiotemporal organization of several signaling pathways (*18*), notably in platelet activation (*22*, *18*). These peculiar phosphoinositides are able to recruit several signaling proteins through specific interaction with protein motifs such as pleckstrin homology domains (PH). As for PtdIns(4,5)P_2, the existence of subcellular pools of PI 3-kinase products has been suggested (*23*). However, the participation of lipid rafts in D-3 phosphoinositide synthesis and storage has not been investigated until now.

In this study, we analyzed the levels of different lipids and lipid second messengers in rafts isolated from resting or thrombin-stimulated platelets. Interestingly, a significant part of thrombin-mediated production of phosphoinositide 3-kinase products and phosphatidic acid concentrated in platelet rafts. Controlled disruption of these microdomains by cholesterol depletion dramatically decreased the production of these second messengers and strongly, but reversibly, inhibited platelet secretion and aggregation induced by either thrombin or collagen.

MATERIALS AND METHODS

Materials. Human α-thrombin was purchased from Enzyme Research Laboratories, and collagen was from Nycomed (Nycomed Arzneimittel, Germany). [^{32}P]Orthophosphate, 5-hydroxy[^{14}C]tryptamine (56.0 mCi/mmol), and enhanced chemiluminescence (ECL) immunoblotting reagents were from Amersham Pharmacia Biotech. (Little Chalfont, U.K.). [γ-^{32}P]ATP (3000 Ci/mmol) was from NEN Life Science Products (Boston, MA). Anti-CD36 monoclonal antibody (MoAb) was from Immunotech (Marseille, France). Anti-Gαq/α_{11} polyclonal antibody was a generous gift from Dr. G. Milligan (Glasgow, U.K.). TLC plates were from Merck (Nogent-sur-Marne, France). Methyl-β-cyclodextrin (MβCD), cholesterol, Triton X-100, and other reagents were from Sigma (Saint Quentin-Fallavier, France) unless otherwise indicated.

Preparation of Platelets. Human platelets were isolated from concentrates obtained from the local blood bank (Etablissement Français du Sang, Toulouse, France) as described previously (*24*). Briefly, they were washed in a washing buffer (pH 6.5) containing 140 mM NaCl, 5 mM KCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM Hepes, 5 mM glucose, and 0.35% BSA (w/v). The same buffer plus 1 mM $CaCl_2$ was added to the final suspension and pH was adjusted to 7.4. In experiments concerning inositol lipid analysis, platelets were labeled with 0.5 mCi/mL [^{32}P]orthophosphate during 60 min in phosphate-free washing buffer (pH 6.5) at 37 °C and stimulated as described previously (*22*).

Isolation of Platelet Rafts. Resting or stimulated platelets (1.5×10^9 platelets in 0.5 mL) were lysed by adding one volume of ice-cold 2× buffer containing 2% Triton X-100 (v/v), 150 mM NaCl, 50 mM Mes, pH 6.5, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM Na_3VO_4, and 2 μg/mL each leupeptin and aprotinin. Subsequent procedures were performed at 4 °C as described previously (*11*, *12*). Briefly, the 1 mL lysate was adjusted to 1.37 M (40%) sucrose by addition of 1 mL of 2.74 M sucrose prepared in Mes-buffered saline (150 mM NaCl, 25 mM Mes, pH 6.5, 1 mM PMSF, and 1 mM Na_3VO_4). A step sucrose gradient [1 M (30%), 0.8 M (25%), 0.6 M (20%), 0.5 M (15%), 0.3 M (10%), and 0.15 M (5%), 1.33 mL each] was layered on top of the 1.37 M (40%) homogenate followed by ultracentrifugation (200000*g*; 4 °C, 16 h) in a Beckman SW41 rotor. Eight fractions of 1.25 mL were harvested with a pipet from the top of the gradient (0.15 M, 5%). Rafts were recovered in fractions 3 and 4.

Alternatively, for analysis of the phosphoinositide content in rafts, the same procedure was used except that the ice-cold 2× buffer contained 0.2% Triton X-100.

Electron Microscopy Analysis. Rafts were isolated from resting or stimulated platelets, as described above in the presence of 1% Triton X-100. Both fractions 3 and 4 were pooled, diluted twice in Mes-buffered saline, and centrifuged (200000*g*; 4 °C, 45 min). The pellet was resuspended in 2% low-melting agarose (prewarmed at 30 °C) and cooled on ice. Rafts were then fixed in glutaraldehyde (1% in PBS) for 1 h, washed twice in PBS, postfixed in 1% osmium tetroxide in water, washed in water, and incubated for several hours in uranyl acetate (0.5% in water). After the samples were washing with bidistilled water, dehydration proceeded by successive incubations in 70% ethanol (15 min), 100% ethanol (3 times, 15 min each), and 100% acetone (5 min). Samples were then embedded in Epon and ultrathin sections (70−90 nm) were examined with a transmission electron microscope at 120 kV (EM420, Philips, Eindhoven, The Netherlands).

Gel Electrophoresis and Immunoblotting. For immunoblotting analysis, proteins from each fraction were precipitated with trichloroacetic acid (5% final concentration) for 30 min at 4 °C, centrifuged (12000*g*; 15 min), washed once in H_2O, and resuspended in electrophoresis Laemmli sample buffer. Immunodetections were performed as previously described (*22*) with the relevant antibodies, peroxidase-conjugated secondary antibody, and the ECL system.

Major Phospholipids and Cholesterol Quantification. Whole platelets or rafts, isolated as described above, were submitted to lipid extraction following the modified procedure of Bligh and Dyer (*25*). Sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine + polyphosphoinositides (PS + PI), and phosphatidylethanolamine (PE) were separated by thin-layer chromatography (TLC) with $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (75/45/12/6 v/v/v/v), identified by iodine vapor staining, in reference to lipid standards, and scraped off. The quantification was performed according

to their phosphorus content (*26*). For cholesterol quantification, lipids were extracted in the presence of 15 μg of stigmasterol (Sigma) as internal standard and injected into a gas chromatograph (Hewlett-Packard), as described previously (*27*).

Analysis of the Fatty Acid Composition of Phospholipids. Lipids were extracted with a $CHCl_3/CH_3OH$ (2/1 v/v) mixture (*28*) from whole platelets or from rafts isolated as described above. SM, PC, PS + PI, and PE were separated and identified by fluorescein staining. PC, PS + PI, and PE were transmethylated for 60 min at 55 °C in CH_3COCl/CH_3OH (5/95 v/v) and SM for 90 min at 100 °C in BF_3 at 14% in CH_3OH. Reactions were stopped by addition of K_2CO_3 (5% w/v) or H_2O for SM. Methylated fatty acids were extracted by addition of isooctane and injected into a gas chromatograph (Dani GC 1000) with a Chrompack fused silica capillary column (5 m length, 0.25 mm inner diameter). Fatty acids were identified according to the retention time of standards and quantified as percentage of total fatty acids. Means were compared with the Kruskal-Wallis nonparametric test by use of the SYSTAT statistical software system. Differences were considered significant at a probability level less than 0.05 ($p < 0.05$).

Phosphoinositide Analysis. For the analysis of [^{32}P]-labeled phosphoinositides in whole cells, reactions were stopped by addition of chloroform/methanol, lipids were extracted following a modified Bligh and Dyer procedure (*25*), and resolved by TLC with $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (80/30/26/24/14 v/v/v/v/v). Spots corresponding to [^{32}P]PtdInsP_2 and [^{32}P]PtdIns(3,4,5)P_3 were visualized by a PhosphorImager 445 SI (Molecular Dynamics, Inc.), scraped off, deacylated by 20% methylamine, and analyzed by high-performance liquid chromatography (HPLC) on a Whatman Partisphere 5 SAX column (Whatman International Ltd., U.K.), as described previously (*22*). [^{32}P]PtdOH was analyzed as described previously (*22*) and quantified by liquid scintillation counting.

To investigate the relative distribution of [^{32}P]-labeled phosphoinositides in sucrose density gradient, platelets were stimulated or not and lysed by addition of ice-cold 2× buffer containing either 2% or 0.2% Triton X-100 as indicated, and rafts were isolated as described above. Lipids were extracted from each fraction of the gradient and analyzed as detailed above.

For lipid kinase assays, fractions 3 and 4 from the gradient were pooled, diluted 3-fold in a buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM PMSF, 1 mM Na_3VO_4, and 2 mg/mL each leupeptin and aprotinin, and rafts were pelleted by ultracentrifugation (200000*g*, 45 min, 4 °C). Rafts were then incubated at 37 °C under shaking for 20 min in 200 μL of kinase buffer (50 mM Tris-HCl, pH 7.4, 1.5 mM dithiothreitol, 100 mM NaCl, 10 mM $MgCl_2$, 100 μM ATP, and 30 μCi of [γ-^{32}P]ATP) in the presence of sonicated vesicles containing PS (40 μg) and the appropriate lipid substrate (20 μg). For diacylglycerol kinase (DAG kinase), phosphatidylinositol kinase (PtdIns kinase) and phosphatidylinositol 4-monophosphate kinase [PtdIns(4)P kinase] assays, the substrate were 1,2-dioleyl-*sn*-glycerol, PtdIns, and PtdIns(4)P, respectively. The reactions were stopped and lipids were extracted and separated by TLC as described above. The radioactive spots were visualized by a PhosphorImager and identified by comparison with standard chromatography profiles. The produced [^{32}P]PtdOH, [^{32}P]PtdInsP, and [^{32}P]-PtdInsP_2 were quantified as described previously (*22*).

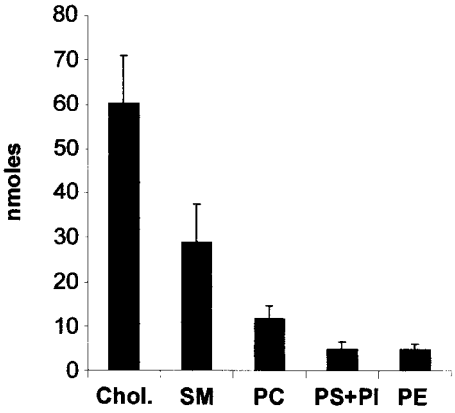

FIGURE 1: Lipid characterization of platelet rafts. Rafts were isolated from resting platelets (1.5×10^9 cells), and their cholesterol and phospholipid composition was analyzed as described under Materials and Methods. Data are expressed as nanomoles and are means ± SD of five independent experiments.

Cholesterol Depletion and Repletion. Cholesterol depletion was performed by incubating platelets (1×10^9cells/mL) for 10 min at 37 °C in buffer A [140 mM NaCl, 5 mM KCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM Hepes, pH 6.5, 5 mM glucose, 0.2% (w/v) BSA, and 6 mM methyl-β-cyclodextrin (MβCD)]. For the cholesterol repletion, cholesterol-depleted platelets were centrifuged and resuspended for 30 min at 37 °C in a buffer containing cholesterol/MβCD complexes prepared as described previously (*29*). Briefly, 200 μL of cholesterol in $CHCl_3/CH_3OH$ (1/2 v/v) were added to a final concentration of 2 mM in 10 mL of buffer A without BSA and containing 5 mM MβCD, under stirring at 80 °C. The solution was homogenized by sonication (60 kHz, 3 × 20 s), filtered through a 0.22 μM filter (Millipore), and then maintained at 37 °C. Platelets were washed and used for aggregation experiments.

Platelet Aggregation and 5-Hydroxytryptamine Secretion Studies. Aggregation was monitored by a turbidimetric method using a dual-channel Payton aggregometer (Payton Associates, Scarborough, Ontario, Canada) with stirring at 900 rev/min at 37 °C (5×10^8 platelets/mL). 5-Hydroxytryptamine secretion was performed as described previously (*22*). Platelets loaded with 5-hydroxy[^{14}C]tryptamine were stimulated for 3 min with thrombin or collagen under stirring at 900 rev/min at 37 °C.

RESULTS

Lipid Characterization of Isolated Platelet Rafts. Rafts isolated from resting platelets were observed as one light-refractive band generally recovered in fractions 3 and 4 of the sucrose density gradient (10−20% sucrose). As previously described (*12*), these fractions contained about 1% of the total proteins but were strongly enriched in CD36 (not shown). The lipid characterization of these platelet microdomains, which is poorly known, was then investigated. Rafts isolated from 1.5×10^9 platelets contained 60.3 ± 10.8 nmol of cholesterol (Figure 1) and the cholesterol/phospholipids molar ratio was 1.2 versus 0.5 in whole platelets. These results are in the range of the values found in other cells (*7*). We found that isolated rafts contained (11.0 ± 0.3) mol % of total platelet phospholipids. SM was the major

Table 1: Comparison of the Fatty Acid Composition in Phospholipids from Rafts and Total Platelets[a]

	PC		PS + PI		PE	
	whole platelets	rafts	whole platelets	rafts	whole platelets	rafts
			Saturated Fatty Acids			
C16:0	23.9 ± 6.0	42.3 ± 13.4[b]	1.7 ± 1.7	9.6 + 5.3[b]	5.0 ± 2.2	15.3 ± 9.9[b]
C18:0	16.2 ± 0.8	25.0 ± 5.0[b]	45.0 ± 2.6	48.8 ± 7.7	19.6 ± 2.6	39.9 ± 16.7[b]
C20:0	1.4 ± 0.4	4.2 ± 1.8[b]	2.0 ± 0.4	3.2 ± 1.2		
			Unsaturated Fatty Acids			
C18:1, n-9	26.4 ± 3.3	17.0 ± 3.9[b]	15.0 ± 2.1	16.6 ± 3.8	6.1 ± 2.1	8.45 ± 4.65
C18:2, n-6	8.9 ± 0.8	3.3 ± 1.1[b]				
C20:4, n-6	12.5 ± 0.8	1.9 ± 1.5[b]	27.9 ± 1.1	11.3 ± 6.7[b]	44.6 ± 2.2	17.8 ± 16.7[b]
C22:4, n-6					4.2 ± 0.1	1.9 ± 2[b]
C22:5, n-3					3.3 ± 0.2	1.9 ± 2.2
C22:6, n-3					3.3 ± 0.9	0.3 ± 0.6[b]

[a] Phospholipids were extracted from rafts or from total platelets and their fatty acid composition was analyzed as indicated under Materials and Methods. Results are expressed as percentage of total fatty acids in each phospholipid class and are means ± SD of four independent experiments. Values represented here concern only the cases in which the percentage, in at least one of the two groups (whole platelets or rafts), is greater than 3. [b] Statistically significant difference ($p < 0.05$) evaluated as described under Materials and Methods.

phospholipid, representing (57.2 ± 5.6) mol % of the total phospholipids in rafts and about (35.0 ± 8.0) mol % ($n = 3$) of total platelet SM. The high level of SM in platelet rafts might be explained by the relatively elevated proportion of this phospholipid in this cell type (*30*).

The fatty acid composition of SM in rafts was not significantly different from that in whole platelets and was mainly composed of saturated fatty acids [behemic (C22:0) > lignolenic (C24:0) > palmitic (C16:0)] and one monounsaturated fatty acid [nervonic acid (C24:1, n-9)]. As shown in Figure 1, small amounts of PC, PS + PI, and PE where also detected in rafts (23, 10 and 10 mol % of phospholipids in rafts, respectively). Interestingly, these phospholipids showed a marked enrichment in the molecular species containing saturated fatty acids, particularly in palmitic (C16:0), stearic (C18:0), and arachidic (C20:0) acids with a concomitant decrease of unsaturated fatty acids such as oleic acid (C18:1, n-9) and arachidonic acid (C20:4, n-6) (Table 1). However, the proportion of unsaturated fatty acids in platelet rafts was not negligible (25% for PC, 34% for PS + PI, and 37% for PE), suggesting that the isolation procedure did not induce an artifactual segregation of restricted species of phospholipids.

Platelet Activation Induced Morphological Changes of Isolated Rafts without Modification in Their Major Lipid Composition. As shown by electron microscopy analysis, the morphology of isolated rafts was changed upon thrombin stimulation (Figure 2A). In agreement with Dorahy et al. (*12*), we observed that rafts from resting platelets formed round vesicles of heterogeneous sizes ranging from 20 to 500 nm (Figure 2A, left panel). However, rafts isolated from stimulated platelets showed a modified curvature and displayed a tendency to aggregate (Figure 2A, right panel). Modifications in lipid composition could explain these phenomena. Thus, we compared the major phospholipids and cholesterol contents in rafts isolated from resting or thrombin-stimulated platelets. However, as indicated in Figure 2B, no significant change was detected.

Significant Thrombin-Induced Production of PtdOH, PtdIns(3,4,5)P_3, and PtdIns(3,4)P_2 in Platelet Rafts. The use of high levels of 1% Triton X-100 in the initial homogenization step could result in a loss of signaling molecules from rafts, such as acylated proteins (*31*) but also phospholipids (*32*).

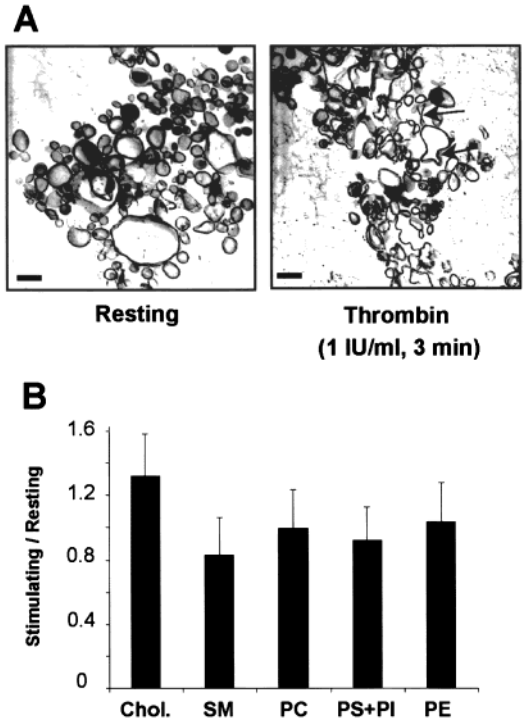

FIGURE 2: Rafts undergo morphological changes upon platelet activation. (A) Rafts were isolated from resting or stimulated platelets and analyzed by electron microscopy. Morphologic changes were observed upon thrombin stimulation (1 IU/mL, 3 min): the arrows indicate a higher curvature of isolated rafts. The scale bar represents 200 nm. Data shown are representative of three independent experiments. (B) Effect of thrombin stimulation (1 IU/mL, 3 min) on the major lipid composition of rafts. The ratios [lipid (nanomoles) in rafts from stimulated platelets/lipid (nanomoles) in rafts from resting platelets] were determined as described under Materials and Methods. Results are means ± SD of five and four independent experiments for cholesterol and phospholipids, respectively.

To avoid this problem, several authors have developed raft isolation procedures in the presence of lower concentrations of Triton X-100 or milder detergents (*11*, *33*−*35*). In agreement, we observed that low percentages of PtdIns, PtdInsP, and PtdInsP$_2$ were recovered in rafts isolated from resting cells with 1% Triton X-100, whereas these percentages significantly increased when rafts were isolated in 0.1% Triton X-100. Therefore, to investigate the dynamic of

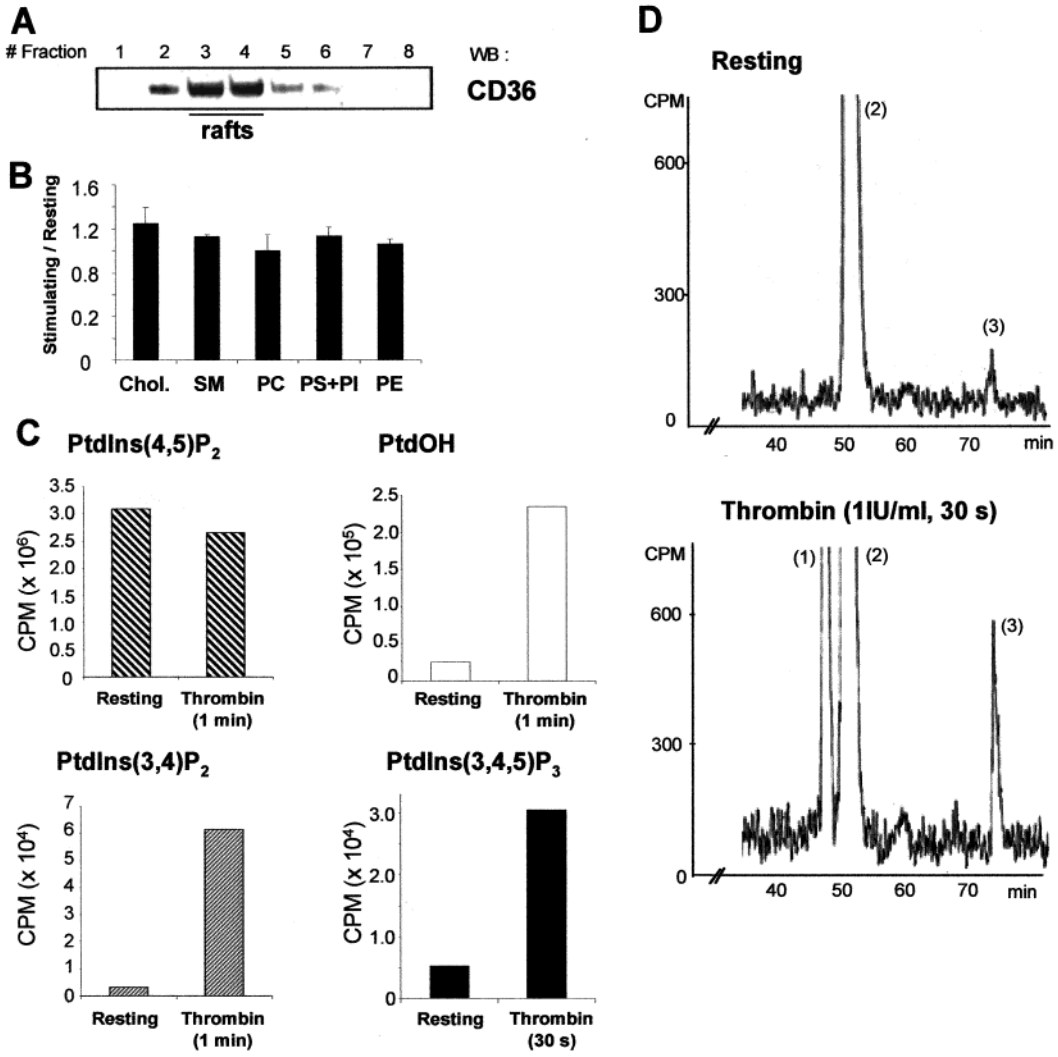

FIGURE 3: Significant thrombin-dependent production of PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, and PtdOH in platelet rafts. (A) Distribution of CD36 in sucrose density gradient after lysis in 0.1% Triton X-100 of 1.5×10^9 platelets. The repartition of CD36 in sucrose density gradient was investigated by immunoblotting half of the proteins from each fraction. (B) Effect of thrombin stimulation (1 IU/mL, 1 min) on the major lipid composition of rafts isolated after lysis in 0.1% Triton X-100. The ratios [lipid (nanomoles) in rafts from stimulated platelets/lipid (nanomoles) in rafts from resting platelets] were determined as in Figure 2B. Results are means ± SD of three experiments. (C) Thrombin-induced production of PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, and PtdOH in rafts. [^{32}P]-Labeled platelets (1.5×10^9 cells) were stimulated by thrombin (1 IU/mL) for the indicated time, lysed in 0.1% Triton X-100, and submitted to ultracentrifugation in a sucrose density gradient as described under Materials and Methods. The lipids were extracted from the gradient fractions (3 and 4) containing rafts and analyzed by HPLC for [^{32}P]phosphoinositides or by TLC for [^{32}P]PtdOH, as described under Materials and Methods. [^{32}P]PtdIns-(3,4,5)P_3 level in rafts was measured after 30 s of thrombin stimulation corresponding to the maximum of its transient production. [^{32}P]-PtdOH and [^{32}P]PtdIns(3,4)P_2 levels were measured at 1 min since they accumulate more slowly. Data are expressed in counts per minute (cpm) and are means of two independent experiments with very similar results. (D) HPLC analysis profiles showing the separation and the relative level of phosphoinositides in rafts (pooled fractions 3 and 4). Radioactivity measured upon thrombin stimulation (30 s) for each phosphoinositide was as follows: (1) PtdIns(3,4)P_2, 6.57×10^4 cpm; (2) PtdIns(4,5)P_2, 2.5×10^6 cpm; (3) PtdIns(3,4,5)P_3, 3.06×10^4 cpm.

signaling molecules within rafts, we used a detergent concentration of 0.1%. The low-density fractions isolated by this method showed a high level of SM (35% of all phospholipids versus 18% in whole cells) and a cholesterol/phospholipid molar ratio of 1.3. The distribution of the different major phospholipids was comparable to the one described in Figure 1 except that the phosphatidylcholine content was slightly higher. More than 95% of the total platelet proteins were recovered in heavy fractions (6−8) (not shown) and the preferential raft localization of CD36, already described in other studies (*12*), was observed (Figure 3A). These features are characteristic of isolated lipid rafts. Moreover, comparison of the major phospholipids and cholesterol content in rafts isolated from resting or thrombin-stimulated platelets did not display significant modification (Figure 3B) as observed with the 1% Triton X-100 procedure. Under these conditions, we found 55% ± 3% of total [^{32}P]-PtdIns(4,5)P_2 in microdomains and upon stimulation its level slightly decreased to 41% ± 2%, suggesting a degradation by a PLC (Figure 3C). Moreover we found a thrombin-dependent production of [^{32}P]PtdOH, [^{32}P]PtdIns(3,4)P_2, and [^{32}P]PtdIns(3,4,5)P_3 in platelet rafts (Figure 3C,D). In resting conditions, [^{32}P]PtdOH and [^{32}P]PtdIns(3,4)P_2 were reproducibly barely detectable either in whole cells or in isolated rafts, whereas low amounts of [^{32}P]PtdIns(3,4,5)P_3 were detected in platelets (Figure 3C,D) and mostly within rafts (not shown).

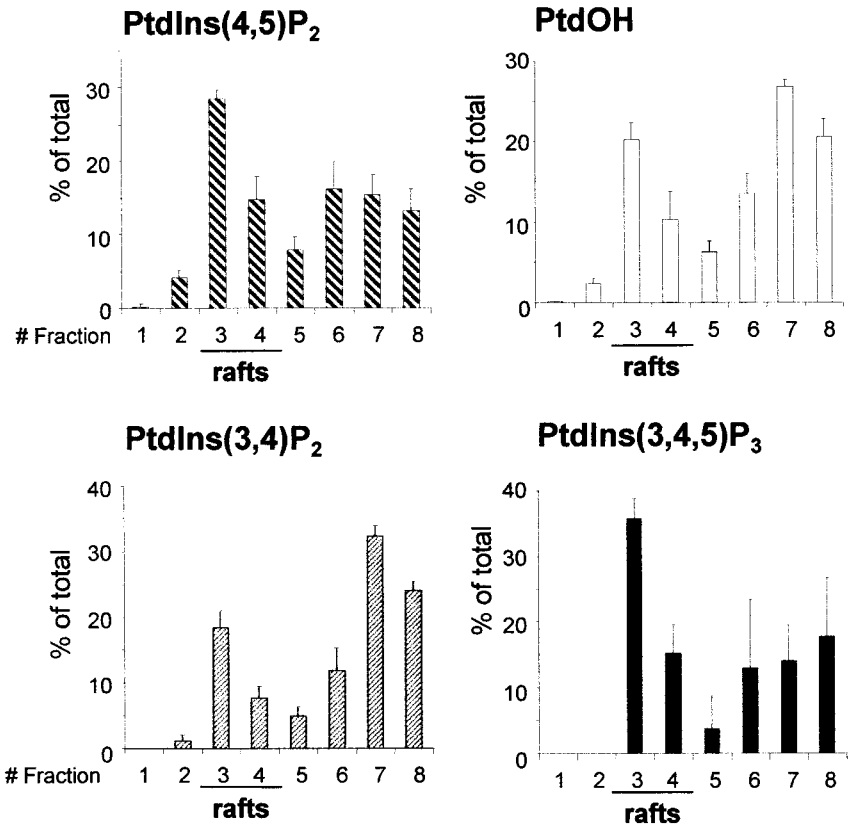

FIGURE 4: Relative distribution of PtdIns(4,5)P_2, PtdOH, PtdIns(3,4,5)P_3, and PtdIns(3,4)P_2 in sucrose density gradient. [^{32}P]-Labeled platelets (1.5×10^9 cells) were stimulated by thrombin (1 IU/mL, 1 min), lysed, and submitted to ultracentrifugation in sucrose gradients as in Figure 3B. Lipids were extracted from each fraction harvested from the top and analyzed as described for Figure 3B and under Materials and Methods. Data are expressed as a percentage of the total and are means ± SD of three independent experiments. Note: A similar distribution was observed for [^{32}P]PtdIns(3,4,5)P_3 in a separate experiment, after 30 s of thrombin stimulation (1 IU/mL).

These results led us to investigate the relative distribution of these lipid second messengers across the sucrose density gradient. Interestingly, a high proportion of [^{32}P]PtdIns-(3,4,5)P_3 (51.1% ± 7.4% of the total) was concentrated in rafts isolated from thrombin-stimulated platelets (Figure 4). Moreover, a lower but significant amount of [^{32}P]PtdIns-(3,4)P_2 (26.0% ± 4.4%) was also detected. 30,6 ± 5.4% of [^{32}P]PtdOH, a lipid known to accumulate in platelets upon activation, mainly as a result of PLC and DAG kinase activities (*36*), was present in rafts (Figure 4). Accordingly, in vitro assay experiments revealed the presence of enzymatic activities involved in the production of these bioactive lipids in rafts isolated from thrombin-stimulated platelets (PtdIns kinase activity, 0.12 pmol/min; PtdIns(4)P kinase activity, 0.35 pmol/min; DAG kinase activity, 5.13 pmol/min). However, the identification and the regulatory mechanisms of these enzymes in platelet rafts are still unknown. This aspect is currently under investigation in our laboratory.

Cholesterol Depletion Disrupts Raft Organization, Inhibits the Production of Second Messengers in Whole Platelets, and Reversibly Impairs Platelet Functions. On the basis of the fact that cholesterol has a potent raft-stabilizing effect, one of the methods widely used to disrupt these microdomains is depletion of cholesterol by methyl-β-cyclodextrin (MβCD) (*20*, *29*, *37*). A dose of MβCD of 6 mM was able to deplete 50% ± 2% of the total platelet cholesterol within 10 min. Under these relatively mild conditions, compared to classically used doses, it was still possible to isolate rafts but their content in cholesterol and CD36 was dramatically reduced (56% ± 4% and 60% ± 9% of decreases, respectively; $n = 3$). Under these conditions, the agonist-mediated production of [^{32}P]PtdOH, [^{32}P]PtdIns(3,4)P_2, and [^{32}P]-PtdIns(3,4,5)P_3 in whole platelets was dramatically inhibited (Figure 5A). Conversely, the levels of [^{32}P]PtdIns(4,5)P_2 were only weakly affected, indicating a role for cholesterol preferentially in the synthesis of lipid second messengers upon thrombin or collagen stimulation. Interestingly, the inhibition of their synthesis by MβCD was more pronounced in rafts than in high-density fractions of the gradient (Figure 5B), suggesting a preferential decrease in their synthesis in the microdomains. The heterotrimeric protein Gq is known to play a critical role in thrombin receptor-dependent signaling (*38*). Therefore, we checked whether Gαq would be recruited to the rafts and if this would be affected by cholesterol depletion. We found a very rapid thrombin-mediated relocation of Gαq in these microdomains (Figure 5C, left panel), which was dramatically affected in cholesterol-depleted platelets (Figure 5C, right panel). In agreement with the inhibition of the production of phosphoinositide-derived second messengers, thrombin- or collagen-induced platelet aggregation (Figure 6A) and secretion (Figure 6B) were strongly inhibited by cholesterol depletion. Complete cholesterol repletion (100% ± 5% of initial concentration, $n = 3$) restored platelet responses (Figure 6A).

DISCUSSION

As in most mammalian cells, it is possible to isolate liquid-ordered-phase lipid rafts from human blood platelets (*12*). As expected, we show here that platelet rafts are enriched

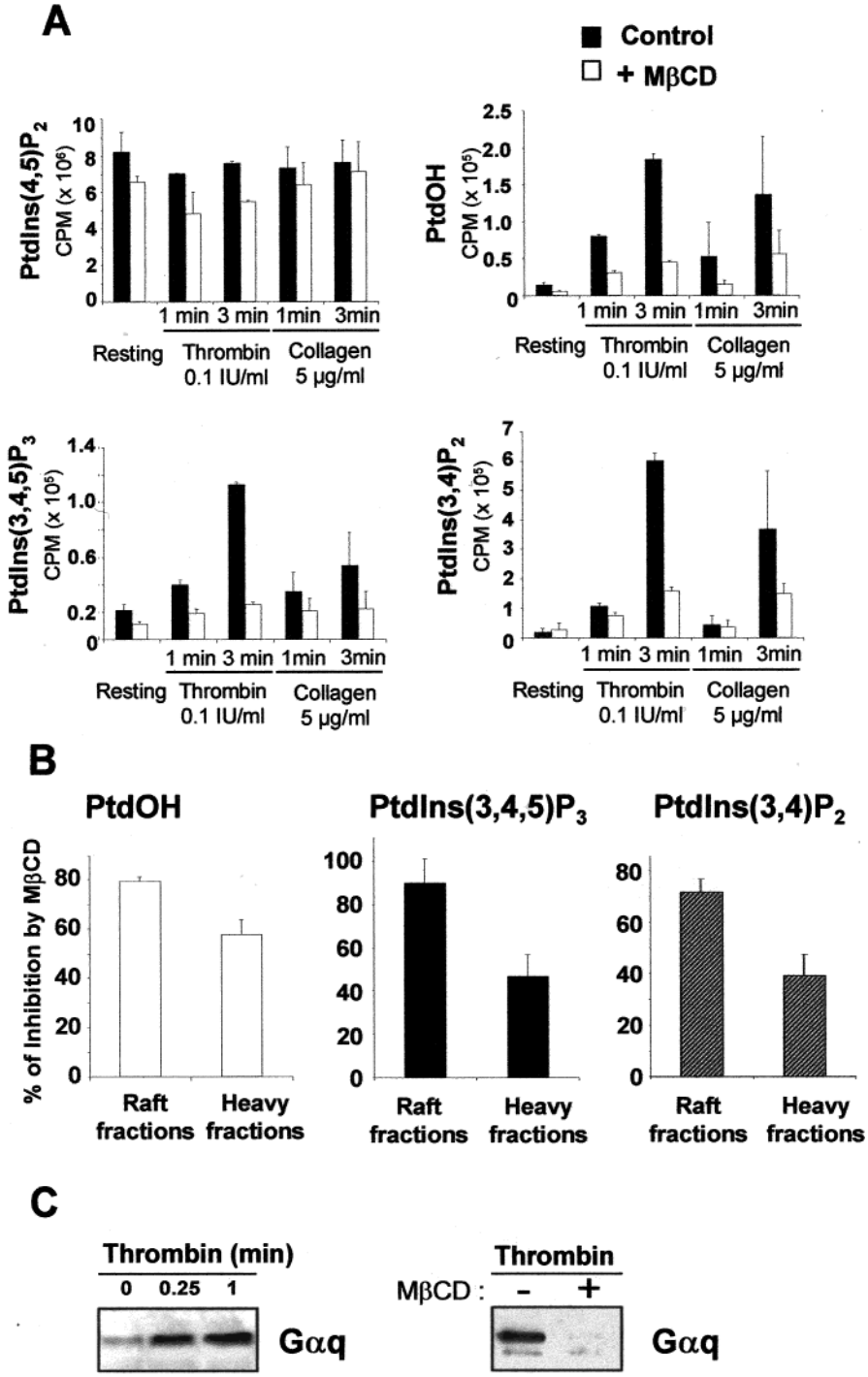

FIGURE 5: Cholesterol depletion severely impairs the productions of PtdIns(3,4)P_2, PtdIns(3,4,5)P_3, and PtdOH induced by thrombin and collagen in whole platelets. (A) [^{32}P]-Labeled platelets were incubated (+MβCD) or not (control) with MβCD (6 mM, 10 min), washed, and stimulated by thrombin (0.1 IU/mL) or collagen (5 μg/mL). The reaction was stopped at the indicated times by addition of chloroform/methanol. The levels of [^{32}P]phosphoinositides and [^{32}P]PtdOH in whole platelets were determined as described under Materials and Methods. Results are means ± SD of three independent experiments. (B) Preferential inhibition of [^{32}P]PtdOH, [^{32}P]PtdIns(3,4,5)P_3, and [^{32}P]PtdIns-(3,4)P_2 production in platelet rafts. [^{32}P]-Labeled platelets were incubated or not with MβCD (6 mM, 10 min), washed, stimulated by thrombin (0.1 IU/mL, 3 min), and submitted to the raft isolation procedure. The levels of [^{32}P]PtdOH and [^{32}P]phosphoinositides in raft fractions (pooled fractions 3 and 4 of the gradient) or in heavy fractions (pooled fractions 6−8 of the gradient) were determined as described under Materials and Methods. Results are means ± SD of three independent experiments. (C) Cholesterol depletion inhibits the thrombin-induced relocation of Gαq in rafts. Rafts were isolated from thrombin-stimulated (0.1 IU/mL) platelets at the indicated times, and the presence of Gαq was then detected by immunoblotting (left panel). The same experiment was performed on rafts isolated from thrombin-stimulated (1 min) platelets pretreated or not with MβCD (6 mM, 10 min) (right panel). Data shown are representative of two independent experiments.

in cholesterol and SM. Indeed, the high acyl chain melting temperature of SM is thought to promote the formation of liquid-ordered domains in the presence of cholesterol (*4*). Platelet rafts also contain some PC, PS, and PE, with a clear enrichment in molecular species containing saturated fatty acyl chains. Thus, besides RBL-2H3 mast cells (*39*), platelets provide the second demonstration of such enrichment. This is an important observation since it has been speculated that

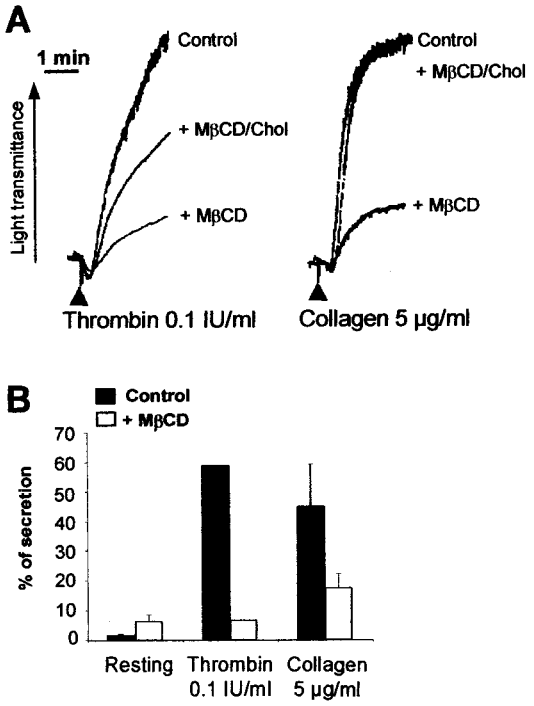

FIGURE 6: Reversible inhibition of collagen- or thrombin-mediated platelet secretion and aggregation by cholesterol depletion. Platelets were incubated (+MβCD) or not (control) with 6 mM MβCD during 10 min in order to deplete 50% of the total cholesterol content. After a washing step they were used for aggregation (A) and secretion (B) assays in response to thrombin (0.1 IU/mL) or collagen (5 μg/mL). To test the reversibility of the inhibition observed, cholesterol-depleted platelets were incubated with MβCD/cholesterol complexes for 30 min in order to restore normal levels of cholesterol, as indicated under Materials and Methods. These platelets (+MβCD/Chol) were then washed and stimulated as described above. Shown in panel A are data representative of three independent experiments with very similar results. In panel B, data are expressed as percentage of secretion and are means ± SD of two or three independent experiments.

the lipid bilayer in rafts is asymmetric with a SM- and glycosphingolipid-enriched outer leaflet and glycerophospholipid-enriched inner leaflet (*3*, *4*). Saturated glycerophospholipids would greatly facilitate the formation of liquid-ordered phase in the inner leaflet of the plasma membrane. The behavior of saturated glycerophospholipids together with the rather high proportion of SM in platelet rafts[2] may explain why we were still able to isolate rafts after 50% cholesterol depletion. Altogether our results suggest that the human platelet membrane provides a favorable lipid composition for raft formation.

Rafts have been shown to play important roles in signal transduction via recruitment of signaling proteins (*3*, *4*), and several pieces of evidence support the presence of lipid mediators in these microdomains (*19*−*21*, *40*). Here, we show that thrombin stimulation induced some changes in the raft morphology suggesting modification of their fluidity. Comparative analysis of their major lipid composition under resting and stimulating conditions did not reveal significant modifications. However, a thrombin-mediated production of key lipid second messengers derived from the PI metabolism occurred in platelet rafts.

[2] Platelets appear rather rich in SM compared to other cells; S. Bodin et al., personal observation.

Distinct metabolic pools of phosphoinositides have been suggested in several cell types, but a detailed spatial picture of the intracellular phosphoinositide metabolism dynamics is still not available. Recent work has indicated that a functional pool of PtdIns(4,5)P_2 is compartmentalized in caveolae (*19*, *20*, *40*) and in rafts of Neuro 2a cells (*21*), suggesting a potential production of DAG and InsP_3 in these microdomains. In agreement, InsP_3 receptor has been found in caveolae (*41*). Here, we show that thrombin treatment leads to the production of PtdOH in these microdomains. In [^{32}P]-labeled platelets, [^{32}P]PtdOH synthesis is a reflection of PLC activation since the main part of DAG produced by PLC is converted into [^{32}P]PtdOH by a DAG kinase, the contribution of phospholipase D being relatively minor (*36*, *42*). The function of PtdOH in platelets is still unknown. In vitro, this lipid has been shown to activate several kinases (*43*) including a type I PtdIns(4)P 5-kinase (*44*). Interestingly, in addition to the canonical pathway leading to PtdIns(4,5)P_2 production and hydrolysis by PLCs, an important fraction of PI 3-kinase products, especially PtdIns(3,4,5)P_3, was also found concentrated in platelet rafts upon thrombin stimulation. By interacting with functional protein domains such as pleckstrin homology domains (PH), D3-phosphoinositides are thought to play a critical role in the spatiotemporal organization of several signaling pathways (*45*). Thus, our results suggest that PI 3-kinase products may participate in targeting signaling proteins to the rafts. In agreement, a recent study has demonstrated a PI 3-kinase-dependent recruitment of the Tec family tyrosine kinase Itk in rafts via its PH domain, which has a high affinity for PtdIns(3,4,5)P_3 (*46*). Moreover, the PtdIns(3,4,5)P_3 5-phosphatase SHIP was recently found to transiently associate with rafts upon B cell activation (*47*), and this may explain the presence of PtdIns(3,4)P_2 in these microdomains. Thus the demonstration of a PtdIns(3,4,5)P_3 concentration in platelet rafts may have implications in the understanding of the organization of signaling complexes in general.

One might think that the unsaturated arachidonic acid of PI would not fit into the raft model as it contains saturated fatty acid phospholipids in the inner leaflet. However, our data support the idea that phosphoinositides, which are quantitatively minor lipids in these microdomains, are not backbone components of rafts but rather signaling molecules probably capable of rapidly interacting with specific targets.

Do rafts play a role in platelet activation? The production of PtdOH, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 in whole cells and particularly in rafts were severely impaired by their disorganization via cholesterol depletion. As a result, platelet secretion and aggregation induced by either thrombin or collagen were dramatically reduced. It is important to note that these cholesterol depletion experiments were done under controlled conditions. Indeed, in addition to the disruption of rafts, dramatic cholesterol depletion may have other effects on membrane structures. Therefore, we have used a rather mild MβCD treatment (6 mM, 10 min) in order to deplete approximately 50% of total platelet cholesterol. A more pronounced depletion induced detectable spontaneous secretion (not shown). Interestingly, under these conditions it was possible to restore platelet functions by cholesterol repletion. Moreover, platelet aggregation induced by high doses of thrombin (>1 IU/mL) was only weakly affected by a 50% cholesterol depletion (not shown), further indicating that

platelet integrity was spared under our experimental conditions. These results suggest that these laterally segregated microdomains allow physical interactions of signaling partners to organize an efficient production of phosphoinositide-derived second messengers in response to thrombin. In agreement, we observed that Gαq, known to play a critical role downstream PAR1, rapidly relocated in rafts upon thrombin stimulation. This relocation was inhibited by cholesterol depletion. As demonstrated with Gαq knockout platelets, αq subunit plays a critical role in platelet activation induced by thrombin (*38*). As suggested by previous studies (*48, 49*), recruitment of Gαq to the rafts may be crucial for the activation of key enzymes involved in the generation of phosphoinositide-derived second messengers required for platelet activation. A correlation between increased cholesterol content or cholesterol/phospholipid ratio and platelet response to ADP, epinephrine, or thrombin in vitro has been observed previously (*15, 16, 50*). These observations suggest that rafts might actually be part of a general process that contributes to efficient platelet activation. The results obtained with collagen suggest that these domains are also involved in the recruitment of signaling molecules such as ITAM-containing proteins or the adapter protein LAT, as previously shown in T cells (*51*). In this respect we have observed that cholesterol depletion led to a dramatic inhibition of PLCγ2 tyrosine phosphorylation induced by collagen (not shown). Furthermore, a critical role of PtdIns(3,4,5)P_3 for full activation of PLCγ2 through binding to its PH domain has been shown in platelets stimulated via the collagen receptor GPVI (*52*) or by FcγRIIA cross-linking (*22*). The production of a pool of PtdIns(3,4,5)P_3 in rafts upon stimulation of these receptors could contribute to the recruitment and/or the stabilization of PLCγ2 in these microdomains where other partners of this enzyme, such as LAT, are concentrated. Activated PLCγ2 may then hydrolyze specifically the pool of PtdIns(4,5)P_2 present in rafts, a hypothesis currently under investigation in our laboratory.

In conclusion, the data obtained in this study, by different approaches, point to the same conclusion and strongly suggest a key role for cholesterol and SM-rich membrane domains in the initiation of signaling mechanisms involved for platelet activation. This is especially the case for the agonist-dependent production of PtdIns(3,4,5)P_3, a key lipid second messenger that appears to be concentrated in lipid rafts. Our results also suggest that modifications in platelet lipid composition leading to changes in the cholesterol/phospholipid ratio, as observed in some dyslipoproteinemias (*17*), may influence raft organization and thereby platelet function, potentially leading to increased risk of thrombosis. Clearly, further work is necessary to characterize the role of rafts in the different phases of platelet activation.

ACKNOWLEDGMENT

Thanks are due to Dr. C. Trumel for her help in preliminaries experiments, Dr. M. Plantavid for helpful discussions, Drs. M. Fukami, K. Missy, M. P. Gratacap, F. Gaits, and A. Ragab for stimulating discussions, and S. Allenbach for technical assistance in fatty acids analysis.

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BI0109313