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Review

Lipid rafts are critical membrane domains in blood platelet activation processes

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Abstract

Among the various hematopoïetic cells, platelets are critical for maintaining the integrity of the vascular system. They must be rapidly activated by sequential and coordinated mechanisms in order to efficiently prevent haemorrhage upon vascular injury. Several signal transduction pathways lead to platelet activation in vitro and in vivo, among them, several are initiated via receptors or co-receptors containing immuno-receptor tyrosine-based activation motifs (ITAM) which trigger downstream signalling like the immune receptors in lymphocytes. However, in contrast to immune cells for which the role of lipid rafts in signalling has largely been described, the involvement of laterally segregated membrane microdomains in platelet activation has been investigated only recently. The results obtained until now strongly suggest that early steps of platelet activation via the collagen receptor GpVI or via FcγRIIa occur preferentially in these microdomains where specific proteins efficiently organize key downstream signalling pathways. In addition, lipid rafts also contribute to platelet activation via heterotrimeric G-protein-coupled receptors. They are sites where the phosphoinositide (PI) metabolism is highly active, leading to a local generation of lipid second messengers such as phosphatidylinositol 3,4,5-trisphosphate. Here, evidence is accumulating that cholesterol-enriched membrane microdomains are part of a general process that contributes to the efficiency and the coordination of platelet activation mechanisms. Here we will discuss the biochemical and functional characterizations of human platelet rafts and their potential impact in platelet physiopathology.

Keywords: Raft; Cholesterol; Signal transduction; Platelet; Phosphoinositide; Hypercholesterolemia

1. Introduction

Platelets play an essential role in haemostasis and provide an attractive model to studying agonist-mediated signal transduction mechanisms. Various natural agonists can activate these enucleated cells, and most of their cognate receptors are well characterized [1]. The platelet plasma membrane is the site of interactions between extracellular stimuli and highly organized intracellular biochemical events supporting platelet activation. The plasticity (fluidity, phase transition, lipid microdomains) of the plasma membrane is essential for the efficiency of signal transduction mechanisms and for physiological platelet responses such as shape change, spreading, secretion, aggregation and clot retraction. The platelet plasma membrane, like the plasma membrane of other mammalian cells, contains a huge number of lipid molecular species that do not simply act to maintain the structural integrity of the cell. It is now becoming clear that individual lipids are also implicated on their own in the regulation of key cellular functions [2]. The ratio between the various lipid species, which differs from one cell to another and can be modified by specific diet, pathological situations or cell stimulation, is an important factor for the formation of lateral domains and for membrane plasticity.

In vitro, platelets can be activated by physiological agonists acting through G-protein-coupled receptors such as thrombin, thromboxane A2 or adenosine diphosphate (ADP) receptors [1]. They also have several key receptors that recruit and activate tyrosine kinase-dependent pathways. This is, for instance, the case for GpVI, a collagen receptor [3], or FcγRIIa, the low-affinity platelet surface receptor for immune complexes [4]. Those two receptors

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initiate a signalling cascade that shares several features with the pathways activated by immune receptors in lymphocytes [3–5]. The clustering of these receptors in the plasma membrane is an essential initial event allowing the recruitment and the spatio-temporal activation of downstream signalling proteins. In immune cells, a number of genetic and biochemical studies support a functional role for lipid rafts in the onset of immune-receptors mediated signalling [6–8]. These membrane microdomains play a critical role for the efficient recruitment and assembly of newly formed multiprotein complexes at the cytoplasmic side of the plasma membrane. Despite the limitations of technologies available to investigate the functions of lipid rafts, recent biochemical and microscopy approaches strongly suggest that they play an important role in platelet activation process. In this paper, we will review the evidences of the existence of rafts in platelets and focus on their potential involvement in different types of agonist-mediated activation. The impact of the membrane cholesterol content on platelet activation mechanisms and its physiopathological implications will be discussed.

2. Characterization of platelet lipid rafts

Separate functional domains with different lipid compositions appear to coexist in biological membranes [8–12]. The liquid-ordered microdomains called lipid rafts are sphingolipid and cholesterol-based structures consisting of very small domains (20–50 nm) of tightly packed lipids displaying lateral mobility [9–11]. Lipid raft formation should therefore be facilitated in membranes rich in cholesterol and sphingolipids since the high acyl chain melting temperature of sphingomyelin promotes the formation of liquid ordered domains in the presence of cholesterol [9,10]. Interestingly, there is a relatively high amount of sphingomyelin in human platelet plasma membrane [13], suggesting a lipid composition in favour of raft formation in these cells. In 1996, Dorahy et al. [14] isolated low density, 1% Triton X-100 resistant glycosphingolipid and cholesterol-enriched membrane microdomains from human platelets at 4 °C. Until now, most of the human platelet raft isolation procedures used were based on the insolubility of these lipid microdomains in Triton X-100 at 4 °C and their ability to float in sucrose density gradient after ultracentrifugation [15,16]. However, several authors [16,17] have modified the Triton X-100 concentration used according to the amount of total platelet proteins in order to avoid a loss of signalling molecules from rafts during isolation as observed in other cells [18,19]. Based on the classic characteristics of lipid rafts, the isolation procedures where low concentrations of Triton X-100 (0.1% or less) are used appear to be valid under conditions where the amount of human platelets per sample is well controlled [16,17,19]. Another nonionic detergent, Brij 58, has also been recently employed for platelet rafts isolation and seems to preserve these microdomains, even when used at high concentrations [19]. Despite the fact that it is always difficult to separate two domains of a membrane with a high yield and without impurities by biochemical methods, the results obtained by different groups using various concentrations of detergent allow one to draw a general characterization of human platelet lipid rafts. When isolated by the classical method (1% Triton X-100 and sucrose gradient) platelet rafts appear as round vesicles of heterogeneous size ranging from 20 to 500 nm [14,16]. They have less than 1% of total platelet proteins, lack caveolin, which is not expressed in these cells, and are highly enriched in the membrane glycoprotein CD36 (Table 1). The impact of CD36 on raft structure is yet unknown and it would therefore be interesting to investigate its role in CD36-deficient platelets (Nak(a)-negative platelet phenotype). Although weakly detected in isolated rafts, other significant plasma membrane glycoproteins such as GPIb, GPIIIa and CD9 are predominantly found in the Triton X-100 soluble fraction of resting platelets [14]. In contrast, the β1 integrin subunit is restricted to the detergent-soluble fraction and may be considered as a non-raft transmembrane marker protein [19]. The transmembrane adapter LAT [17,20], as well as the tyrosine kinases of the Src family, notably Lyn [14,15], are present to a high level in rafts isolated from resting platelets. As observed in other hematopoïetic cells, platelet rafts are enriched in the GM1 ganglioside, known to preferentially partition into liquid ordered domains [17] (Fig. 1). The cholesterol/phospholipid molar ratio is 1.2 in rafts versus 0.5 in whole platelets and indicates a clear cholesterol enrichment [16] (Fig. 1). Sphingomyelin is the major phospholipid of isolated rafts, representing about 57 mol% of the total raft phospholipids and about 35% of total platelet sphingomyelin. The fatty acid composition of sphingomyelin in rafts is not significantly different from that in whole platelets and is mainly composed of saturated fatty acids (behenic>lignoceric>palmitic) and one monounsaturated fatty acid (nervonic acid) [16]. Small amounts (<5%) of phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine are detected in isolated rafts but these phospholipids display a marked enrichment in saturated fatty acids, particularly in palmitic, stearic and arachidic acids with a concomitant decrease of unsaturated fatty acids [16]. It is noteworthy that when low concentrations of Triton X-100 are used to isolate rafts, the amount of phosphatidylcholine increases but the levels of the other lipids do not change significantly [Bodin et al., personal observation].

Besides RBL-2H3 mast cells [21], human platelets are another example of enrichment in molecular species containing saturated fatty acyl chains in rafts. This enrichment is consistent with a model in which the lipid bilayer in rafts is asymmetric with sphingomyelin and glycosphingolipids enriched in the outer leaflet and saturated glycerophospholipids enriched in the inner leaflet [7,9,21]. In the presence of cholesterol, saturated glycerophospholipids would facilitate

Table 1
Signalling proteins and lipid second messengers found so far in rafts isolated from resting or activated human platelets

	Resting	FcγRIIa activation	GpVI activation	Thrombin activation	References
GpIIbIIIa	−/+(1)	?	?	?	(1) [14]
GpIb	+	?	?	?	[14]
FcγRIIa	+	+++	?	?	Bodin et al., Thromb. haemost. (in press)
GpVI	+	?	+++	?	[17,19]
Fcγ-chain	?	?	+++	?	
LAT	++	+++ (1)	+++ (2,3)	++ (4)	(1) Bodin et al., Thromb. haemost. (in press) (2) [17,19] (3,4) Bodin et al., unpublished data
p85α	−	+	?	+	Bodin et al., unpublished data
PLCγ2	−	+(1)	+ (2)	?	(1) Bodin et al., Thromb. haemost. (in press) (2) [19]
SLP76	−	?	+	?	[19]
pp60src	−	?	?	+	[14]
Lyn	++	++ (1)	++ (2)	++ (3)	(1) Bodin et al., Thromb. haemost. (in press) (2) [17] (3) [15]
Fyn	+	?	?	+	[15]
CD36	+++(1)	+++(2)	?	+++ (3)	(1) [14] (2) Bodin et al., Thromb. haemost. (in press) (3) [16]
αi	−	+ (1)	?	++ (2)	(1) Bodin et al., unpublished data
αq	−	?	?	++ (2)	(2)[16]
PtdIns(4,5)P_2	++ (1)	++ (2)	?	++ (1)	(1) [16]
PtdIns(3,4)P_2	−	++ (2)	?	++ (1)	
PtdIns(3,4,5)P_3	−	++ (2)	?	++ (1)	(2) Bodin et al., Thromb. haemost. (in press)
PtdOH	−	++ (2)	?	++ (1)	

the formation of a liquid ordered phase in the inner leaflet of the plasma membrane. For example, in vitro experiments have shown that a mixture of monounsaturated phosphatidylcholine and cholesterol is partially Triton X-100 insoluble at low temperature [22]. This is an important issue because, due to the potential lipid asymmetry of rafts, one leaflet might be more resistant to detergent than the other.

Recently, using a particular fluorescence microscopy technique, Fourier transform infrared spectroscopy (FTIR), Gousset et al. [23] have obtained evidence that rafts are present in the plasma membrane of human platelets at physiological temperatures and can aggregate upon stimulation. Although the small size of rafts and platelets is a limitation for the clear visualization of these microdomains

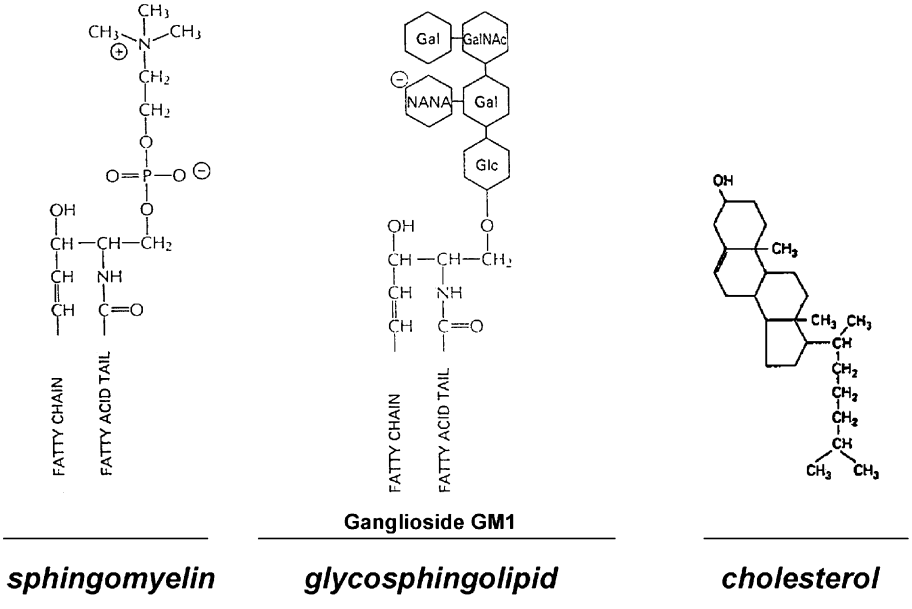

Fig. 1. Representation of three of the quantitatively major and representative lipids found in rafts isolated from human platelets.

by microscopy in general, it is now necessary to develop these approaches to confirm the biochemical data.

Altogether, these results indicate that, like other hematopoïetic cells, platelets contain lipid rafts that are likely to be highly dynamic microdomains of the plasma membrane, if we refer to their fast lateral movements and their capacity to aggregate.

3. Phase transition and raft aggregation during platelet activation

A number of studies have shown that cold temperatures (below 18 °C) can induce platelet activation [24–26] and, for this reason, platelet concentrates must be stored at 22 °C in blood banks. Tablin et al. [27] have investigated whether low temperatures would initiate signalling events through modification of the fluidity of the platelet plasma membrane. These authors have demonstrated that upon cooling through the phospholipid membrane phase transition (15 °C), platelets change their shape with emission of pseudopodial projections [27]. Using diI-C18 (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate), a fluorescent dye expected to preferentially partition into ordered lipid domains, Gousset et al. [23] have shown by FTIR that lipid raft-like domains aggregate during the initial steps of platelet activation upon chilling (Fig. 2). Interestingly, this lipid phase separation upon cooling appeared to be a reversible process [23]. Using the same technique, it was shown that upon stimulation by physiological agonists, such as thrombin or collagen, lipid rafts also coalesce at 37 °C [23]. Methyl-β-cyclodextrin (MβCD), which efficiently removes cholesterol from the plasma membrane and thus disrupts lipid raft organization, inhibited these events. These observations support previous results suggesting a redistribution of cholesterol within the plasma membrane upon collagen stimulation [28]. Thus, this dynamic process might represent a critical mechanism for the early steps of platelet activation under physiological conditions. These results are in agreement with observations showing that, in several hematopoietic cells, clustering of small lipid rafts into larger aggregates participates in the formation of signalling platforms [7,8].

4. Implication of lipid rafts in GpVI-mediated platelet activation

Platelet adhesion to the exposed subendothelium at sites of tissue damage is one of the first critical steps in the complex processes required for the prevention of bleeding [29]. At high shear forces, in damaged arteries or arterioles, adhesion of platelets to von Willebrand's factor (vWf) through the surface glycoprotein GpIb-IX-V is an important initial mechanism of the repair process. At lower flow rates, collagen and fibrinogen can support adhesion of platelets. The integrin α2β1 is a collagen receptor [30] involved in platelet adhesion to most forms of collagen under flow conditions. Besides this initial stage of adhesion via α2β1, another collagen receptor, GpVI, restricted to megakaryocytes and platelets [31], is strongly involved in the platelet activation process [5]. GpVI initiates a signalling cascade comparable to the one activated by immune receptors [3]. This activating pathway will lead to the secretion of molecules such as ADP, which will further stimulate platelets, and to the activation of the αIIbβ3 integrin, allowing

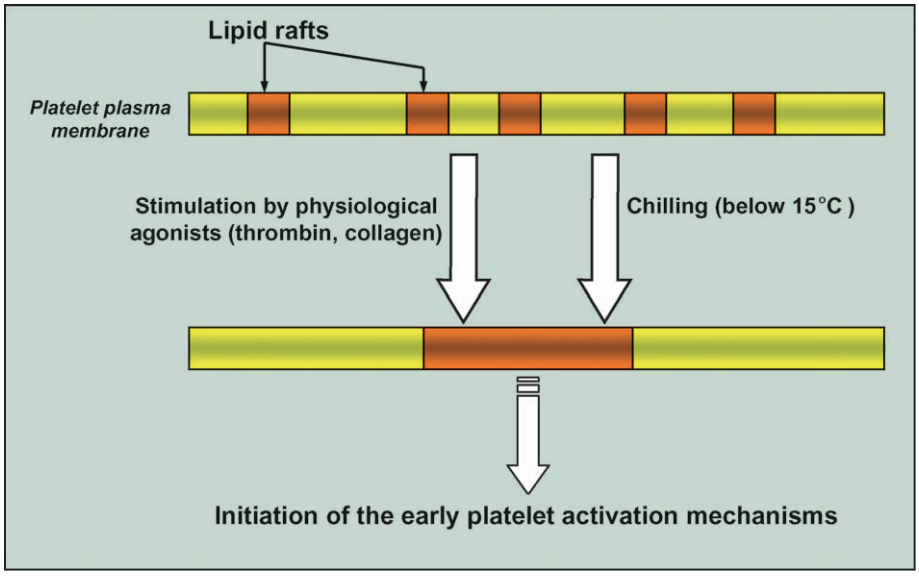

Fig. 2. Chilling- as well as thrombin- or collagen-induced aggregation of platelet lipid rafts. Several studies [14–17,23] strongly suggest that, in resting platelets, lipid microdomains in liquid ordered phase, displaying the characteristics of rafts, have a homogeneous distribution in the platelet plasma membrane. These lipid microdomains appear to coalesce in the early stages of platelet activation induced by chilling or by physiological agonists.

fibrinogen binding and in turn interaction between platelets and finally aggregation [3,5]. Genetic and biochemical studies have demonstrated that, like immune receptors, functional GpVI requires the formation of a multimeric complex [3,5,32]. The Fcγ-chain, an immuno-receptor tyrosine-based activation motif (ITAM)-containing transmembrane protein, plays a critical role in GpVI-mediated platelet activation [5,33,34]. Fcγ-chain is phosphorylated by the Src kinases, Lyn and Fyn, allowing the recruitment and the activation of the tyrosine kinase Syk. Syk will, in turn, phosphorylate key adapter molecules like LAT and SLP-76 and regulate critical signalling proteins such as PLCγ2 [5,35–37].

The role of lipid rafts in GpVI-mediated platelet activation was first indirectly suggested by Boesze-Battaglia et al. [28] who observed a redistribution of cholesterol within the platelet plasma membrane upon collagen stimulation. Watson et al. [5] also obtained biochemical evidence suggesting a role of lipid rafts in GpVI-dependent platelet activation. These observations were recently confirmed by Wonerow et al. [19] and Locke et al. [17] using both human platelets and the basophilic RBL-2H3 cell line expressing GpVI. Locke et al. [17] demonstrated that GpVI is not present in rafts isolated from resting cells but is massively recruited to these microdomains following receptor stimulation. The optimal recovery of clustered GpVI in rafts is observed with an isolation procedure using very low concentrations of Triton X-100. Using Brij 58 as a detergent for raft isolation, Wonerow et al. [19] found a considerable amount of GpVI in rafts from resting platelets and no major change in its distribution upon stimulation. This discrepancy suggests a rather weak association of GpVI with lipid rafts that may be well preserved by Brij 58 and only partially by Triton X-100 when rafts are isolated from resting platelets. Locke et al. [17] suggest that the Fcγ-chain is required for the stable association of GpVI with lipid rafts upon activation [17], but the molecular basis of this mechanism is still unknown. Phosphorylated Fcγ-chain is exclusively found in lipid rafts, but this phosphorylation is not required for the association of GpVI with the microdomains [17]. The transmembrane domain of the Fcγ-chain may be critical for the specific targeting of oligomerized GpVI receptors. An arginine residue in the transmembrane domain of GpVI can form a salt bridge with an aspartic residue in the transmembrane domain of Fcγ-chain to stabilize the complex [34]. However, it is still unclear whether the Fcγ-chain directly drives the force to target GpVI in the microdomains or whether it uses intermediate molecules.

These studies suggest that phosphorylated Fcγ-chain specifically organizes signalling events in lipid rafts where other proteins such as the docking protein LAT will subsequently coordinate downstream mechanisms. This is consistent with the fact that cholesterol depletion using MβCD strongly affects the tyrosine phosphorylation events induced by GpVI triggering [5, Bodin et al., Thromb. Haemost. (in press)]. The phosphorylation of the Fcγ-chain by Lyn may specifically occur in platelet rafts since active Lyn is selectively enriched in these microdomains [15]. LAT is known to concentrate in T lymphocyte rafts where it plays a central role in the organization of functional signalling complexes downstream of immune receptors [19,38,39]. Palmitoylation of cysteines 26 and 29 of LAT is responsible for the targeting of this adapter molecule in T lymphocyte lipid rafts [40]. Once phosphorylated (mainly by Syk in platelets and Zap-70 in T lymphocytes), LAT can associate with several proteins, including the p85α subunit of phosphoinositide 3-kinase (PI 3-kinase), Grb2, PLCγ2, Gads and SLP-76 [19]. In platelets, LAT has a major role in the tyrosine phosphorylation and the activation of PLCγ2 downstream of GpVI and is required for normal platelet activation via collagen or collagen-related peptide [35]. However, despite a lack of platelet response to GpVI, LAT-deficient mice have no bleeding phenotype. This is in contrast with the dramatic bleeding problems observed in SLP-76- [36] or Syk- [33] deficient mice. These two proteins, which are not classical raft proteins, play a critical role in platelet activation via GpVI [37,41,42]. Wonerow et al. [19] found that a small fraction of tyrosine-phosphorylated Syk and SLP-76 is recruited to these domains upon GpVI triggering. However, since the majority of these phosphotyrosyl proteins are present outside the rafts, the authors suggest either a transient association of these molecules with the microdomains or a dissociation of the complex upon cell lysis. A participation of Syk and SLP-76 in biochemical events occurring outside lipid rafts is also likely.

Thus, strong arguments are in favour of a role of lipid rafts in GpVI-mediated platelet activation but several points need to be clarified to better understand the sequence of molecular events taking place in these microdomains. An interesting aspect would be to investigate whether the other collagen receptor, α2β1, can function independently of lipid rafts.

5. A role for lipid rafts in a critical step of FcγRIIa-mediated platelet activation

FcγRIIa is the only class of immune Fcγ receptor expressed in platelets and is involved in several pathologies, including heparin-associated thrombocytopenia (HIT) [43–47]. HIT occurs in patients under heparin therapy who develop IgG against heparin–platelet factor 4 complexes at the platelet surface. Binding of Fc fragments of this IgG on FcγRIIa leads to the activation of a signalling pathway that contributes to thrombosis and/or rapid destruction of platelets. In this process, secreted ADP is a crucial co-factor that positively modulates platelet activation induced by FcγRIIa stimulation [48,49].

Upon cross-linking of FcγRIIa, either by successive additions of the nonactivating specific anti-FcγRIIa monoclonal antibody IV-3 and $F(ab')_2$ fragments or by addition of

HIT sera and heparin, this receptor becomes rapidly tyrosine-phosphorylated in its ITAM motif, probably via Lyn. The phosphorylated ITAM is a docking site for SH2 domain-containing signalling proteins, including the kinase Syk, involved in the tyrosine phosphorylation of LAT and PLCγ2 [50–52]. The phospholipase is responsible for the production of the second messengers inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG), key signalling events for FcγRIIa-dependent platelet secretion and aggregation [52].

Recently, our group has investigated the role of lipid rafts in the spatio-temporal organization and the integration of FcγRIIa-dependent signalling events [Bodin et al., Thromb. Haemost. (in press)]. Upon cross-linking, FcγRIIa massively relocates in lipid rafts where the kinase Lyn and the adapter LAT are among the major phosphotyrosyl proteins. Moreover, upon stimulation of human platelets by HIT sera, PtdIns(3,4,5)P_3 is preferentially produced in lipid rafts. This lipid, whose production is dependent on co-activation by secreted ADP via its P_2Y_{12} receptor, appears to be required to specifically recruit PLCγ2 and/or to stabilize its presence in platelet rafts [52,53]. In human platelets activated through FcγRIIa, as well as GpVI, tyrosine phosphorylation of PLCγ2 is required but not sufficient to activate the enzyme since the early synthesis of the PI 3-kinase product, PtdIns(3,4,5)P_3, is an obligatory step in this mechanism [52,53]. PtdIns(3,4,5)P_3 can directly interact with the PH domain and/or the SH2 domain of PLCγ2 and participates in the adequate localization of this phospholipase [52] close to its substrate, the PtdIns(4,5)P_2 [54]. In human platelets, the generation of phosphatidic acid (PtdOH) via phosphorylation of DAG is a good indication of PLC activation [55,56]. In agreement, an important proportion of PtdOH was detected in rafts isolated from platelets activated via FcγRIIa. This production was blocked by PI 3-kinase inhibitors or antagonists of the P_2Y_{12} ADP receptor. In

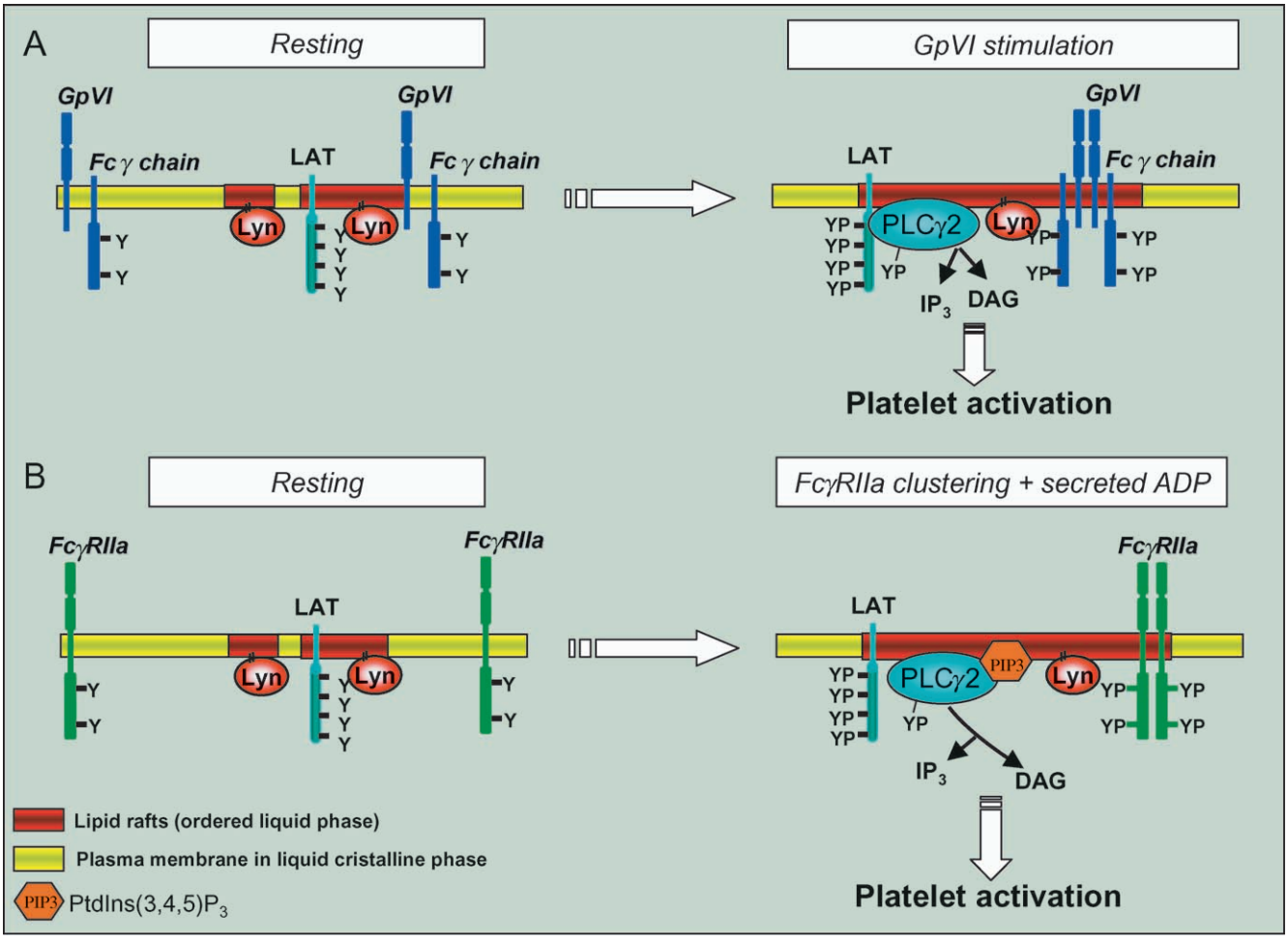

Fig. 3. Involvement of lipid rafts in GpVI and FcγRIIa-mediated platelet activation. (A) Upon stimulation, a considerable amount of GpVI molecules is present in lipid rafts [17,19] together with the ITAM-containing Fcγ-chain which appears to be exclusively tyrosine-phosphorylated in these cholesterol-rich membrane domains [17]. (B) Upon clustering, FcγRIIa associates with lipid rafts in its tyrosine-phosphorylated form. The other major tyrosine-phosphorylated proteins in rafts are the tyrosine kinase Lyn and the adapter LAT. Converging signalling pathways from FcγRIIa and P_2Y_{12} ADP receptor [53] are required for the production of a high proportion of PtdIns(3,4,5)P_3 in lipid rafts, which in turn, allows the recruitment and the activation of PLCγ2. Thus, lipid rafts appear as membranous sites where critical steps of the FcγRIIa-mediated signalling pathway are organized leading to efficient platelet activation.

order to assess the role of rafts in FcγRIIa-mediated platelet activation, these microdomains were destabilized by cholesterol depletion using MβCD. In this context, it is important to note that the conditions to use MβCD have to be carefully adjusted according to the cell type. For instance, 5 mM MβCD can deplete more than 45% of total human platelet cholesterol within 5 min. Under conditions where 40% cholesterol is depleted, a slight secretion can be observed and no perturbation of shape change, secretion and aggregation is noted in response to thrombin at concentrations above 0.5 IU/ml [Bodin et al., Thromb. Haemost. (in press)]. This controlled cholesterol depletion induced a significant disruption of platelet rafts and impaired the association of FcγRIIa with the remnants of lipid rafts without affecting significantly the tyrosine phosphorylation events induced by FcγRIIa clustering. However, the production of PtdIns $(3,4,5)P_3$ and the activation of PLCγ2 were strongly inhibited in whole platelets. This inhibition was reversible as complete cholesterol repletion restored PLCγ2 activation. These results highlight the role of the lipid second messenger, PtdIns$(3,4,5)P_3$, in the recruitment and/or the stabilization of PLCγ2, a key protein of the signal transduction cascade in lipid rafts (Fig. 3).

The fact that the tyrosine phosphorylations initiated by FcγRIIa cross-linking are only weakly sensitive to cholesterol depletion and raft disruption, while collagen-mediated tyrosine phosphorylations are highly sensitive, suggests a differential role of rafts in the organization of these two platelet activating pathways. Partial disruption of rafts may be sufficient to inhibit the phosphorylation processes in the case of receptors that function as coordinated complexes, such as TCR or collagen GpVI receptor, which require the ITAM containing Fcγ − chain to signal. In the case of FcγRIIa, which possesses its own ITAM, the early phosphorylation mechanisms are less sensitive to raft disruption and these laterally segregated microdomains are rather essential to organize key downstream events such as PI 3-kinase and PLCγ2 activation.

6. G-protein coupled receptor signalling in lipid rafts

The role of lipid rafts in G-protein coupled receptor-mediated signalling is still poorly characterized in general. In human platelets, thrombin is one of the best known physiological agonist and acts through the seven transmembrane domains containing receptors PAR1 and PAR4 [57]. We have observed that thrombin stimulation leads to morphological changes of isolated platelet rafts. This is correlated with a rapid association of the α subunit of the heterotrimeric G proteins Gq [16] and Gi [Bodin et al., our unpublished data] with the microdomains and the production of lipid second messengers in rafts [16]. Cholesterol depletion with MβCD reversibly impairs these biochemical events as well as platelet secretion and aggregation induced by low doses of thrombin (<0.3 IU/ml). These observations are in agreement with the results of Gousset et al. [23] showing by FTIR that cholesterol- and sphingolipid-rich domains aggregate during the initial steps of platelet activation induced by thrombin. However, at high concentrations of thrombin, we observed that 40% to 50% cholesterol depletion does not significantly affect platelet aggregation. Taken together, these results suggest that lipid rafts are mainly involved in the mechanism of activation induced by the high affinity thrombin receptor (PAR1).

7. Phosphoinositide (PI) signalling pathway in platelet rafts

PIs are quantitatively minor phospholipids (about 10% of total phospholipids) but they have a highly active metabolism and play a crucial role in many mechanisms of cell regulation [54]. Several PIs are specifically involved in the control of the spatial and temporal organization of key signalling pathways leading to cell proliferation or survival, rearrangement of actin cytoskeleton or intracellular vesicle trafficking. Various cellular agonists control the activity and/or the recruitment of kinases, phosphatases or phospholipases involved in the tight control of the intracellular level of polyphosphoinositides. This metabolism is highly active in platelets and plays a central role in these cells.

The remarkable feature of PIs, which can be rapidly synthesized and degraded in discrete membrane domains or subcellular structures, places them as accurate regulators and integrators of dynamic mechanisms of cell regulation [54]. They can directly and specifically interact with functional protein modules such as pleckstrin homology (PH), FYVE or Phox (PX) domains, thus allowing membrane targeting of several signalling proteins. Evidence suggests that distinct metabolic pools of PIs exist in various cell types, but we have only a rough idea of the spatial organization of intracellular PI metabolism dynamics.

In nucleated cells [58–60], as well as in platelets [16], cholesterol- and sphingolipid-rich membrane domains appear as site where the PI metabolism is highly active, suggesting a production of DAG and $InsP_3$ in these microdomains. One might think that the unsaturated arachidonic acid of PIs 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 PIs, which are quantitatively minor lipids in these microdomains, are not backbone components of rafts but rather signalling molecules able to rapidly interact with specific targets. In this context, an important issue is to understand how these PIs are kept concentrated in lipid rafts. The implication of specific PI-binding proteins such GAP43, MARCKS or CAP23, also called Pipmodulines, has recently been suggested [61]. These proteins interact with PtdIns$(4,5)P_2$ and are proposed to retain or mask this PI in raft-like membrane microdomains, thus regulating its accessibility to other regulatory proteins. For example, proteins involved in the

dynamic organization of the actin cytoskeleton, such as proteins belonging to the WASP–Arp2/3 complex actin nucleation machinery, could also be recruited to lipid rafts through their binding to this PI [54,62]. In this respect, it is interesting to note that PtdIns(4,5)P_2 has been shown to induce actin-based movements of raft-enriched vesicles through the WASP–Arp2/3 pathway [63].

In addition to PtdIns(4,5)P_2, our study reveals that PtdIns(3,4,5)P_3 is largely produced and/or concentrated in these microdomains upon platelet stimulation and plays an important role in recruiting or stabilizing PLCγ2 [52,53] and other enzymes containing a PH domain such as the tyrosine kinases BTK and Tek [54]. The presence of PtdIns(3,4,5)P_3 in lipid rafts might be a general mechanism in activated hematopoïetic cells and could explain, for example, some of the roles of this second messenger in lymphocytes. One of the limiting steps in the production of PtdIns(3,4,5)P_3 in rafts may be the access of PtdIns(4,5)P_2 to activated PI 3-kinase. As seen above, this access could be partly modulated by Pipmodulin proteins.

8. Variation of plasma membrane cholesterol concentration in platelet physiopathology

The question of the role of cholesterol in platelet activation was addressed in the 1970s, at a time when the notion of lipid rafts had not been yet developed. Several studies showed that cholesterol content and platelet function were altered in some dyslipoproteinemias [64–67]. These changes in cholesterol concentration are likely explained by molecular mechanisms established in the megakaryocyte [68]. Indeed, de novo cholesterol synthesis does not occur in platelets and the cholesterol exchange process, which can take place in plasma, was shown not to be responsible for a net uptake of cholesterol into platelets [68]. A good correlation was observed between increased cholesterol content or cholesterol/phospholipid ratio and increased platelet responsiveness in vitro [68–70]. For example, platelets from Type IIa hypercholesterolemia patients have a higher membrane cholesterol content [66] and display increased reactivity to collagen and ADP [65,68]. However, the cholesterol content may not be the only abnormality in platelets from patients with genetic dyslipoproteinemias. Therefore, studies in which the cholesterol content of normal platelets was altered by cholesterol–phospholipid dispersions in vitro were undertaken. Incubation of normal platelets with cholesterol-rich liposomes resulted in a similar hyperreactivity [68–70] and a reduced inhibitory effect of prostaglandin E1 [68]. Thus, manipulation of the cholesterol content of platelets in vitro has somehow mimicked the hyperreactivity of platelets from individuals with Type IIa hypercholesterolemia. Conversely, other studies have shown that platelets with a cholesterol/phospholipid ratio lower than 0.51 have a decreased reactivity to epinephrine and ADP [71,72]. These results indicate that this particular lipid plays a critical role in early platelet activation. Together with our recent observations, they suggest that hypercholesterolemia may be a supplementary factor of risk to develop thrombosis in patients suffering of HIT.

The fact that changes in cholesterol affect several platelet responses suggests that this lipid is part of a general process contributing to an efficient platelet activation. One can consider that these results are consistent with a role of cholesterol in the organization and stabilization of lipid rafts and thus in the control of raft-mediated events. However, changes in cholesterol concentration will probably primarily modify raft organization but also, to some extent, the membrane fluidity. Therefore, these observations do not permit unambiguous conclusions at this stage but suggest that a modification in platelet cholesterol/phospholipid ratio may influence raft organization and therefore platelet functions. This modification could contribute, in some cases, to an increased risk of thrombosis in patients with hypercholesterolemia.

9. Conclusions

Altogether, the results commented above strongly suggest that lipid rafts are highly dynamic platelet membrane structures involved in critical signalling mechanisms. Like in T cell activation [73], the membrane compartmentalization appears to be required for efficient platelet activation via receptors or co-receptors containing an ITAM motif. However, due to the limits of the techniques used until now, many aspects of the role of lipid rafts in platelets remain uncertain. The use of new techniques such as real-time imaging should help to appreciate the dynamic of platelet rafts and their contribution to a specific mechanism. The development of raft isolation procedures at physiological temperature with detergents such as Brij 98 [74] could also help to preserve their native state and to identify the proteins found in these microdomains.

In this context, Shrimpton et al. (personal communication, ISTH XVIIIth Congress, Paris 2001) suggested that members of the GpIb-IX-V complex (palmitoylated GpIX and GpIbβ) localize in platelet rafts. Moreover, GpIb has been shown to associate with FcγRIIa [75] possibly in the microdomains. This important point requires an extensive investigation which may provide a better understanding of the role of rafts in platelet biology.

Although the activation of Syk and the tyrosine phosphorylation of PLCγ2 by αIIbβ3 do not depend on rafts [19], it will be important to investigate the role of these microdomains in the late platelet responses such as clot retraction. The interaction of actin cytoskeletal elements with lipid rafts has been described in lymphocytes [76] and could represent an important aspect in platelet activation processes. Indeed, the actin cytoskeleton is strongly reorganized during platelet activation and may participate to maintain the lateral assembly of these microdomains in the membrane, possibly

via PtdIns(4,5)P_2 [54,61–63]. The contractile forces required for clot retraction could take place through cytoskeleton-associated lipid rafts. In this respect, it would be interesting to investigate whether the major platelet integrins can associate with lipid rafts during the irreversible phase of platelet aggregation.

Finally, we have obtained some evidence that different populations of rafts may coexist or be generated during platelet activation. The identification of the specific functions of these different microdomains should help to better understand how the dynamic organization of the plasma membrane participates in the control of platelet biology in normal and pathological situations.

Note added in proof

Two recent studies highlight the role of rafts in platelet activation: Shrimpton et al. J. Exp. Med. 2002, 196, 1057–66 and J. Biol. Chem. 2003, in press.

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