Involvement of platelet cytoskeleton and plasma membrane in the expression of procoagulant activity

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**Involvement of platelet cytoskeleton
and plasma membrane
in the expression of procoagulant activity**

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Involvement of platelet cytoskeleton and plasma membrane in the expression of procoagulant activity

Proefschrift

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, Prof. Dr. F.I.M. Bonke,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen,
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door

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Life is what you make it
Talk Talk

Face it, enjoy it, control it
B.Luiwammes

Voor mijn dierbaarsten,

Sylvia
Hugo
Pieter
Aernout

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CHAPTER 1

GENERAL INTRODUCTION

This thesis will deal with cellular and molecular mechanisms which are involved in the loss of transbilayer lipid asymmetry in the platelet plasma membrane when platelets become procoagulant upon activation. As a general introduction, the major subjects of the research described in this thesis will be discussed in detail to set the scope of the following chapters. First, the structure and function of human platelets will be covered. Second, the properties of calpain, an endogenous Ca^{2+} -dependent protease, will be discussed. Third, regulation of transbilayer lipid asymmetry in lipid model systems and in the erythrocyte will be addressed. Finally, various aspects of platelet procoagulant activity will be reviewed.

The purpose of this general introduction is not merely to provide sufficient background information to understand the following chapters, but it is also meant to provide adequate depth to place the following chapters in a broader perspective, and to provide detailed entrance to the literature. At the risk of supplying redundant information, I hope it will aid those, interested in but not familiar with the fields of platelet biochemistry, calpain, transbilayer lipid asymmetry, and platelet procoagulant activity.

STRUCTURE AND FUNCTION OF THE HUMAN PLATELET

Blood platelets are highly reactive cells which participate in the arrest of bleeding upon vessel wall injury by the formation of a hemostatic plug. Normally, platelets circulate in the blood stream for 8-11 days as quiescent cells. Upon vessel wall injury, blood platelets instantly adhere to the uncovered subendothelial fibrils, change shape, and secrete their granular contents which in turn results in stimulation of other circulating platelets to aggregate on the adherent layer to form a platelet plug. This primary hemostatic plug will prevent leakage of blood from the damaged vessel in normal hemostasis, or may obstruct the blood stream within the vessel in thrombotic processes and in the pathology of severe atherosclerotic lesions. The primary platelet plug is consolidated by fibrin to form a stable final hemostatic plug. Fibrin is formed upon initiation of the coagulation cascade, a process which is dramatically accelerated when activated platelets become procoagulant. Finally, contractile mechanisms in platelets lead to retraction of the clot.

The platelet has become a well studied cell, and many aspects of cell biology and cellular biochemistry are best understood in the platelet. The following subjects will be discussed in more detail: (1) platelet morphology, (2) platelet responses and stimuli, (3) composition and function of the platelet cytoskeleton, (4) structure and composition of the platelet plasma membrane, and (5) platelet signal transduction.

Platelet morphology

Platelets are anucleate cytoplasmic fragments originating from megakaryocytes in the bone marrow [524, 566]. They circulate as disk-shaped smooth cells, with an equatorial diameter of 2-4 μm and a thickness of about 1 μm . Platelet ultrastructure has been studied extensively by White and co-workers [694, 695, 697, 699]. The main features of platelet morphology are shown in Fig. 1. Based on electron micro-

Fig. 1. Morphology of the unstimulated discoid platelet. Schematic representation of structural features as observed by thin-section electron microscopy. The various components are described in the text.

scopic investigations, the platelet can be divided into four distinct regions: peripheral zone, sol-gel zone, organelle zone, and internal membrane systems.

The peripheral zone constitutes the platelet plasma membrane, which is extensively invaginated into the cell interior to form the open canalicular system. The plasma membrane is covered by an exterior coat (glycocalyx), which is thicker and more dense than that of other blood cells. The exterior coat is rich in glycoproteins, which play an important role in various platelet functions. The glycoproteins carry terminal sialic acid residues which confer a large negative surface charge to the platelet. At the cytosolic side of the plasma membrane a system of filamentous elements is found between the membrane and the circumferential band of microtubuli. This filamentous submembrane region will be discussed in more detail as membrane skeleton.

The sol-gel zone consists of a circumferential band of microtubuli, located in the equatorial plane. The microtubule band consists of a single polymer of tubulin, coiled to form 10 to 20 circles [441]. Close to the microtubule band amorphous precursors of the cytoskeleton can be found, which will polymerize into organized cytoskeletal structures (sol-gel transition) during platelet activation.

In the cytoplasm of platelets various secretory vesicles can be detected, which are randomly distributed in unstimulated platelets but become centralized upon activation. The electron-dense bodies contain serotonin and a non-metabolic (storage) pool of Ca^{2+} , ATP, and ADP, in the form of high-molecular weight aggregates [146]. The less dense granules are the most abundant organelles, and can be divided into α -granules, lysosomes, and peroxisomes. α -Granules are protein storage sites, which contain a number of non-enzymatic proteins [308, 458]. Some of these proteins partici-

Fig. 2. Morphology of the stimulated platelet. Schematic representation of structural features as observed by thin-section electron microscopy. Characteristic events of platelet stimulation are: agonist-receptor interaction (1), granule centralization (2), and fusion of granules with the open canalicular system (3) resulting in release of their contents (4). Further details are described in the text.

pate in coagulation, like fibrinogen, factor V, and heparin-binding protein (platelet factor 4). Other proteins support adhesion of platelets to the subendothelium, like fibronectin, thrombospondin, and von Willebrand factor. α -Granules also contain β -thromboglobulin, albumin, several protease inhibitors, and platelet-derived growth factor, which stimulates the proliferation of arterial smooth muscle cells and fibroblasts. The lysosomes hold a number of acid hydrolases, such as hyaluronidase, α -N-acetylglucosaminidase and β -glucuronidase [38, 290]. Besides secretory vesicles, the platelet cytoplasm contains also a small number of mitochondria and abundant glycogen granules.

Two membrane systems can be detected in platelets: the open canalicular system (OCS) and the dense tubular system (DTS). The OCS forms many channel-like invaginations of the plasma membrane deep into the platelet interior. These channels serve to enlarge the surface area of the plasma membrane available for membrane fusion during the release reaction, and convey the substances released by the internalized granules to the surrounding fluid. The DTS is derived from the smooth endoplasmic reticulum of the parent megakaryocyte and forms a tubular network, not in contact with the OCS. The DTS is the major storage site for Ca^{2+} , which is actively accumulated and which can be released into the cytoplasm during platelet activation [138]. At the surface of the DTS, formation of thromboxane A_2 from arachidonic acid takes place [357], and possibly also the *de novo* synthesis of fatty acids and phospholipids [96].

Platelet responses and stimuli

The initial reaction in hemostasis following injury to vascular endothelium is adhesion of platelets to subendothelial structures [238, 266, 279, 373, 551, 600, 601] which comprise microfibrils of elastin, basement membrane-like amorphous material and collagen fibrils. Of these structures, collagen is required to achieve full release and aggregation following adhesion. At high shear rates, von Willebrand factor (vWf) is necessary for adhesion. After binding of vWf to subendothelium, it can be recognized by a specific platelet receptor.

Most stimuli cause the platelet to change shape. This change involves the formation of very fine pseudopodia, followed by a gradual departure from the disk shape until it becomes a spiny sphere with much broader pseudopodia (Fig. 2) [440]. The formation of pseudopodia is dependent on a reorganization of the cytoskeleton, which is also responsible for the centralization of granules during shape change. Following granule centralization, platelets secrete the contents of their granula [602]. This release reaction involves discharge of granular contents into the open canalicular systems after fusion with the plasma membrane. The contents of α -granules and dense-bodies are most easily released, while secretion from lysosomes is delayed and requires the more potent stimulatory agonists [290].

Aggregation of platelets requires extracellular Ca^{2+} and fibrinogen. When platelets are activated without stirring they do not aggregate but shape change and release still occur [727]. Aggregation results when dimeric fibrinogen binds to receptors on two different platelets [167, 279]. Binding sites for fibrinogen are induced during platelet stimulation. Aggregation is reinforced by a lectin-like association between platelet-bound fibrinogen and thrombospondin, a large protein that is secreted from α -granules and remains attached to platelets [371, 439].

Activated platelets accelerate clot-formation in various coagulation assays [53, 399, 676]. They may trigger the intrinsic coagulation pathway by activating factor XII adsorbed on the surface of ADP-activated platelets [584]. Collagen-stimulated platelets may directly activate factor XI in the absence of factor XII_a [676]. Most powerful is the platelet procoagulant activity, which involves the exposure of negatively charged phospholipids at the platelet surface to provide a catalytic surface on which various coagulation factors interact [732].

In the final stage of hemostatic plug formation, the platelet/fibrin clot retracts [115]. This process is dependent on the contractile activity of the platelet cytoskeletal apparatus, which is intercellularly connected via transmembrane glycoproteins and fibrin.

Platelets can be stimulated by a variety of ligands, which can be understood in terms of temporal and spatial processes occurring after damage of the vessel wall. Initially, platelets will be activated *in vivo* by collagen and other

fibrillar components of the subendothelial matrix, by ADP released from damaged cells, and by thrombin which is formed rapidly from circulating prothrombin at sites of vessel wall damage. These platelets secrete ADP, and will release cyclooxygenase products like endoperoxides and thromboxane A₂ (TxA₂) [8, 319, 534, 537]. These compounds provide a positive feedback (autocoids), and, together with thrombin, activate other platelets not in direct contact with the subendothelial matrix, resulting in increased thrombus size. This positive feedback system is probably limited by haemodynamic factors which limit the effective concentration of activators at the periphery of the thrombus, and by stimulation of endothelial cells by thrombin and ADP to produce prostacyclin, which is a powerful inhibitory platelet agonist [246, 247, 667]. Of the stimulatory agonists, thrombin, platelet-activating factor (PAF), and TxA₂ are the most potent, promoting the full range of platelet responses [101, 169, 263, 534, 667, 727]. Stimulation of platelets by collagen is dependent on secondary formation of TxA₂, which may be related to the relatively small number of in direct contact with collagen [8, 447, 557, 680]. ADP is able to provoke shape-change and a partial, reversible, aggregation (primary aggregation), but is dependent on TxA₂ formation for expression of release and irreversible (secondary) aggregation [534]. Other stimulatory agonists are epinephrine [583], serotonin [169], and vasopressin [395a]. Epinephrine and serotonin are weak activators, dependent on TxA₂ formation for induction of secretion and aggregation, and probably play a role by amplifying the responsiveness to other activators [94]. Vasopressin is able to induce all platelet responses. In vivo, any role for vasopressin and PAF in the activation of human platelets seems unlikely in view of their low abundance [532]. All platelet responses require adequate levels of metabolic ATP [288, 289, 661, 662].

Composition and function of the platelet cytoskeleton

Cytoskeletal proteins constitute a major part (20 %) of total platelet proteins, and play important roles both in stimulatory processes, as well as in many, if not all, platelet responses. They have been extensively reviewed elsewhere [139, 204, 205, 214, 271, 378, 382, 384, 658, 693, 695, 696, 698]. The building blocks are the thin filaments, polymers of actin. The organization of the cytoskeleton[†] is dependent on the regulation of actin polymerization and on the organizing effects of actin-binding proteins. Mechanical force is generated by controlled interaction between actin filaments and myosin filaments.

Monomers of actin (42 kDa[#]) can self-associate in a noncovalent interaction to form actin filaments. The initial phase of polymerization is the formation of nuclei, which is greatly accelerated in the presence of cations, or can be induced by nucleating proteins that complex more than one actin monomer [332, 507]. Actin filaments have a defined polarity, which is demonstrated by decoration with heavy meromyosin. The two ends of actin filaments are identified in this way as the barbed end and the pointed end. Longitudinal growth of actin filaments is favored at the barbed end. Polymerization of actin in platelets is regulated by profilin and gelsolin. Profilin (16 kDa) forms a 1:1 complex with actin monomers, thus inhibiting the polymerization of actin [405]. In platelets monomeric actin is associated entirely with profilin [405]. Gelsolin (90 kDa) binds to the barbed end of actin filaments in the presence of micromolar free Ca²⁺, resulting in fragmentation of actin filaments [82, 383]. Gelsolin remains attached to the barbed ends of the short filaments and acts as a capping protein.

Platelets are abundant in high-molecular weight actin-binding proteins, which orchestrate the functional changes in organization of the cytoskeleton during platelet activation [reviewed in: 136, 332, 507, 615, 616]. Filamin (250 kDa) and talin (235 kDa) each comprise about 5% of total platelet protein. Both proteins are elongated rods which form head-to-head dimers, and are involved in linking actin filaments to membrane proteins. Talin links bundles of actin filaments to the cytoplasmic domain of the fibronectin receptor in adhesion plaques

[†] The name cytoskeleton is used in an aspecific manner to describe any three-dimensional organization of cytoskeletal proteins within a cell.

[#]All molecular weights indicated, refer to reduced proteins.

[292]. In platelets, the glycoprotein (GP) IIb/IIIa complex constitutes the fibronectin receptor [203, 226, 486a]. Thus, talin may link actin filaments to GP IIb/IIIa in platelets. Filamin is an actin-crosslinking protein [382], which links actin filaments to GP Ib [207, 472, 608]. These properties suggest that filamin is responsible for the organization of a submembrane cytoskeletal structure in platelets [204, 206]. Platelet α -actinin (100 kDa) exists as a heterodimer, and induces crosslinking and bundle formation of actin filaments in the absence of Ca^{2+} , which is different from muscle α -actinin [363]. In unstimulated platelets α -actinin is concentrated at the barbed end of actin filaments, adjacent to the plasma membrane [153]. In activated platelets, it is concentrated in pseudopodia [522]. There is evidence that α -actinin has an affinity for specific lipids, i.e. diacylglycerol [84, 85]. Platelet spectrin (240 kDa) resembles brain spectrin (fodrin) instead of erythrocyte spectrin [215]. Just like in the erythrocyte, it has a peripheral location, and the proteins that could link spectrin to the plasma membrane (ankyrin and protein 4.1) are also present in platelets [148, 204]. However, since the amounts of spectrin/ankyrin/protein 4.1 in platelets are very small with respect to other actin-binding-proteins (filamin, talin, α -actinin), they cannot be important elements of a peripheral cytoskeletal structure in platelets.

The generation of mechanic force within platelets results from interaction of myosin filaments with actin filaments, which stimulate the Mg^{2+} -ATPase activity of myosin [506]. Platelet myosin represents about 5% of total platelet proteins, and, like skeletal muscle myosin, consists of two heavy chains of 200 kDa, two light chains of 20 kDa and two light chains of 16 kDa [3]. The two heavy chains form a rod with a globular head to which the light chains are attached. Platelets may contain two different pools of myosin: one cytosolic and one membrane bound [430, 492]. Bipolar filaments of myosin have not been observed in intact platelets, but can be induced in solubilized platelets [454]. Polymerization of myosin is enhanced when myosin light chain (MLC) kinase phosphorylates the 20 kDa light chains of myosin, or when the concentration of Mg^{2+} -ATP is reduced [576]. In intact platelets, where high concentrations of Mg^{2+} -ATP are present, phosphorylation/dephosphorylation is the major mechanism to regulate the interaction

between myosin and actin filaments. Other factors which regulate the interaction between myosin and actin filaments are tropomyosin, troponin, and caldesmon [116, 615]. Platelet tropomyosin (31 kDa) forms a heterodimer [132], and is believed to prevent the binding of myosin to actin filaments, like skeletal muscle tropomyosin, in defined regions of the cell [204, 489, 604]. Troponin has been isolated from pig platelets, and shown to participate in the modulation of actin-activated myosin-ATPase [639]. Caldesmon (80 kDa) binds to actin filaments in the presence of Ca^{2+} , and binds to calmodulin in the absence of Ca^{2+} [520]. Thus, caldesmon has been suggested to regulate the binding of myosin to actin filaments. Caldesmon is also found in platelets, showing a similar Ca^{2+} -dependent behavior as smooth muscle caldesmon [165].

Platelet cytoskeleton research nowadays discriminates between cytoplasmic skeleton and membrane skeleton [29, 206, 207, 210, 446, 471, 482, 546]. These terms can be defined both operationally and morphologically. The cytoplasmic skeleton refers to the precipitate obtained after low-speed centrifugation of cells solubilized with 1% Triton X-100. The remaining soluble fraction contains also cytoskeletal proteins, which can be isolated by high-speed centrifugation. In addition, the high-speed fraction contains many of the plasma membrane glycoproteins, which are complexed to actin filaments by actin-binding proteins [206]. Therefore the high-speed fraction is taken to represent a special structure, the membrane skeleton, a network of short actin filaments lining the plasma membrane and coupled to transmembrane proteins via actin-binding proteins. Most likely, the cytoplasmic and membrane skeleton only reflect structures which remain stable in Triton X-100 and which have lost part of the associated proteins. From a morphological point of view, both thin section [204, 210] and quick-freeze deep-etch electron microscopy [446, 471] clearly reveals the existence of a cytoskeletal structure lining the cytosolic face of the platelet plasma membrane, supporting the membrane skeleton as a functional concept.

In unstimulated discoid platelets, 40 to 50% of the total platelet actin is in a filamentous form [211, 301]. Free actin monomers in platelets are associated with profilin [405]. Profilin is not a very effective inhibitor of actin polymerization once polymerization nuclei

(like the barbed ends of actin filaments) are present [332]. Thus, it appears that profilin acts more as a buffer, and that nuclei are capped. Several candidates for barbed-end capping proteins exist in platelets. One of them is gelsolin [382]. Other candidates are proteins that link actin filaments to the plasma membrane, because polymerization appears to occur in a submembranous location and because the barbed ends of actin filaments in activated platelets are adjacent to the plasma membrane [440]. Talin is such a link-protein that has been found to cap actin filaments in purified systems [123]. α -Actinin is another candidate for a capping protein [332, 616]. The cytoplasmic skeleton of unstimulated platelets contains, besides actin filaments, the actin-binding proteins: filamin, α -actinin, and tropomyosin [239, 301, 541, 546, 717, 718]. However, most of the cytoskeletal proteins in unstimulated platelets are

present in the membrane skeleton, which contains, beside short actin filaments, several actin-binding proteins (filamin, talin, myosin, and α -actinin), and several membrane glycoproteins (GP Ib, GP IX, and as minor components: GP Ia, GP IIa, and GP 250) [206, 207, 253]. Through its linkage to GP Ib/IX complex the membrane skeleton determines the organization of this complex within the membrane [210], and regulates the ability of GP Ib to bind to vWf [119]. Another component of the platelet cytoskeleton are the microtubules [139, 698]. Tubulin (55 kDa) is a major platelet protein, and is polymerized into a single coil that is wound tightly on itself at the periphery of the platelet underneath the membrane skeleton. The peripheral microtubule coil lies in the equatorial plane and is involved in the maintenance of the discoid shape of the platelet.

Stimulation of platelets results in an increase in actin polymerization, comprising 70–80% of the total actin [90, 301]. The critical step in inducing actin polymerization within platelets may be the release of a barbed-end capping protein, or the formation of new nuclei [204]. In addition, dissociation of actin from profilin may be an important step, which has been related to the metabolism of polyphosphoinositides [369, 370]. During platelet stimulation the organization of actin filaments dramatically changes from loose networks into bundles of actin filaments in pseudopodia, and rings of actin filaments surrounding centralized granules [68, 214, 309, 379, 380, 388, 480, 693, 695, 696]. Simultaneously, changes in the distribution of filamin, α -actinin, and myosin take place, which is evidenced by the increase of these proteins in the cytoplasmic skeleton, and a concomitant decrease of the same proteins in the membrane skeleton [206, 207]. Myosin becomes phosphorylated and as a result associates with actin filaments [213], thus generating the tension required for centralization of granules. Centralization of granules is related to, but not dependent on, centripetal reorganization of the microtubule coil [698]. The contractile force for granule centralization is generated by actomyosin. In addition, elongated actin filaments bundle through the action of filamin and α -actinin, and support the outgrowth of pseudopodia [440]. Phosphorylation of filamin may provide a mechanism for modulating its affinity for actin [724]. The glycoprotein IIb/IIIa complex in the plasma mem-

Fig. 3. Tentative model for the involvement of the cytoskeleton in the formation of pseudopodia during platelet activation. In unstimulated platelets (A) filamin is associated with GP Ib. Upon stimulation of platelets, phosphorylation of filamin and polymerization of actin results in the outgrowth of a bundle of actin to which GP IIb/IIIa becomes crosslinked through a linkage protein which may be talin, in view of its involvement in cell-cell contact [see text]. Interaction of the actin bundles with myosin (bottom) provides the force for clot-contraction. Adapted from [231].

brane becomes linked to the actin filament bundles [481, 688] (Fig. 3) in the pseudopodia, thereby permitting, via its binding to fibrinogen, tension to be transferred from the cytoskeleton to the extracellular fibrin network.

Structure and function of the platelet plasma membrane

The platelet plasma membrane is the best studied and probably most complex organelle of the platelet. As a general feature of biological membranes it is composed of a lipid bilayer which contains both transmembrane as well as peripheral proteins. It functions as a permeability barrier through which transport of solutes and ions is carefully controlled by transport proteins and ion-channels. It is the locus of receptor proteins by which a variety of ligands may trigger or inhibit platelet activation. In addition, it contains many proteins involved in signal transduction by generation of second messengers. The plasma membrane is covered at its cytosolic side by a network of cytoskeletal proteins, which may modulate the properties and organization of membrane proteins, and which contributes to its elastic properties. Moreover, the platelet plasma membrane plays an intricate role in the expression of platelet responses: during shape-change it is involved in pseudopod formation, during the release reaction granules fuse with the plasma membrane, and the formation of a procoagulant platelet surface is dependent on changes in lipid distribution. The components of the platelet plasma membrane and their function has been reviewed elsewhere [46, 110, 138, 350, 467, 498, 607, 730, 731].

The lipid bilayer contains 70% phospholipids by weight, while the remainder predominantly consists of cholesterol plus a small amount of glycolipids. The cholesterol content of the plasma membrane is two to three times higher than that of intracellular membranes [186, 243, 358, 418]. Five major phospholipid classes have been identified in human platelets: phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SphM), phosphatidylserine (PS), and phosphatidylinositol (PI) [79, 96, 98, 195, 243, 358, 396, 418, 494, 569]. PE consists for about 70% of plasma-

logen-PE [79]. The phospholipids are asymmetrically distributed over the bilayer [96, 98, 494, 568]. The outer leaflet of the plasma membrane contains almost all SphM and is enriched in PC, while the cytosolic leaflet contains most, if not all, of the PS and PI and is enriched in PE. For further details about phospholipid transbilayer asymmetry is referred to the section concerning platelet procoagulant activity. Not only the phospholipids but also their acyl chains are asymmetrically distributed over the plasma membrane. The major part of esterified arachidonic acid in the plasma membrane is present in the cytosolic leaflet [494], where it is incorporated in PC, PE and PI at the sn2 position. Though PE and PI are enriched in arachidonic acid to a higher degree than PC, most of the arachidonic acid is present in PC due to its high abundance [396, 358].

The plasma membrane has a unique enzymatic composition different from intracellular membranes (DTS), as is revealed by separation of platelet membranes by gradient centrifugation and free flow electrophoresis [138]. Adenylate cyclase is probably exclusively located in the plasma membrane. Phospholipase A₂, diglyceride lipase, cyclooxygenase, and thromboxane synthase, the machinery for production of TxA₂, is predominantly located in intracellular membranes (DTS). The distribution of lipoygenase is less extreme. Ca²⁺/Mg²⁺-ATPase activity seems only present in intracellular membranes, suggesting that extrusion of cytosolic Ca²⁺ via the plasma membrane does not play a significant role in intracellular Ca²⁺ homeostasis.

The plasma membrane is coated on the exterior with an electron dense glycocalyx, representing the carbohydrate rich domains of membrane glycoproteins. These glycoproteins function as receptors for plasma-borne stimuli, and play a role in the adhesion of platelets to the subendothelium and in platelet aggregation. Their receptor function and their absence as a cause of many congenital platelet disorders has lead to extensive investigations with respect to isolation and characterization of platelet membrane glycoproteins [reviewed in: 41, 43, 46, 108, 109, 110, 117, 120, 230, 350, 391, 466, 467, 468, 469, 495, 497, 498, 500, 608].

Glycoprotein I_a (167 kDa) is a relatively minor platelet surface component, which contains several intrachain disulphide bonds [110]. There is evidence that GP I_a is linked to the

platelet cytoskeleton via filamin [212]. Findings with a patient defective in GP Ia [455] suggest that GP Ia functions as collagen receptor.

Glycoprotein Ib is the major sialoglycoprotein, consisting of an α -chain (150 kDa) and a β -chain (26 kDa), which both are transmembrane proteins linked together by disulphide bonds [496]. The extracellular part of the α -chain is easily hydrolysed by various proteases, resulting in the release of a large water-soluble fragment, termed glycocalicin [268, 473, 653]. The cytoplasmic segment of the β -chain can be phosphorylated [67, 609, 711]. In situ, GP Ib is present in a 1:1 complex with GP IX (22 kDa) [42, 44, 121, 174, 209, 419, 700]. In unstimulated platelets GP Ib is linked to the membrane skeleton via filamin [206, 207, 472, 608, 609]. GP Ib contains a binding site for thrombin [225, 268, 412, 631]. In addition, GP Ib is involved in adhesion of platelets to the subendothelium as a binding site for vWf adsorbed to collagen [120].

Glycoprotein IIa (150 kDa) is a less studied protein. It contains several intramolecular disulphide bonds, and it may be complexed to GP Ia [149, 353, 562]. The function of GP IIa is unknown, but there are indications that it is structural similar to a family of proteins called "very late activation" antigens [502].

Glycoproteins IIb and IIIa are major components of the platelet plasma membrane where they are distributed as heterodimer complexes, which play an important role in platelet aggregation and reinforcement of adhesion [reviews: 117, 498]. GP IIb/IIIa exist also as an intracellular pool in the membrane of α -granules [686, 709]. GP IIb is composed of two disulphide-linked subunits, α (132 kDa) and β (25 kDa). GP IIIa (110 kDa) is a single polypeptide chain crosslinked by multiple intrachain disulphides [175]. Formation of the GP IIb/IIIa complex is dependent on Ca^{2+} , since chelation of Ca^{2+} dissociates the complex [202, 221, 592]. Morphological investigations [91] showed that the complex consisted of a globular head (GP IIb) and a rod-like tail (GP IIIa). In unstimulated platelets the GP IIb/IIIa complex is evenly distributed in the plasma membrane [686]. Activation of platelets results in clustering of the complexes [19, 387], and increased binding to the cytoskeleton [501, 689]. The major function of the GP IIb/IIIa complex is to form a binding site for adhesive proteins, like fibrinogen [403, 491], fibronectin [203, 226, 486a] and vWf [400,

488]. Binding these proteins and linking them to the actinomyosin of the platelet cytoskeleton provides the mechanism for clot retraction. A common recognition mechanism has been proposed for these adhesive proteins: they all contain the sequence Arg-Gly-Asp, which is recognized by GP IIb/IIIa [226, 278, 503, 523]. In addition to the tripeptide sequence, GP IIb/IIIa may have a second class of binding sites for adhesive proteins [326, 362, 561]. The binding site for fibrinogen on GP IIb/IIIa is dependent on intimate association of both glycoproteins [310], and hence on the presence of Ca^{2+} . In unstimulated platelets the binding site for fibrinogen is not expressed. Its expression requires conformational changes during platelet activation [118, 120a, 594]. The regulation of the expression of the fibrinogen binding site on GP IIb/IIIa is still unclear, though it has been suggested to be dependent on phospholipase C activation [591], and on interaction of GP IIb/IIIa with the cytoskeleton [35, 491]. Binding of vWf, secreted by platelets, to GP IIb/IIIa on activated platelets may be involved in the reinforcement of platelet aggregation [488]. In addition to its role in platelet aggregation, some findings suggest that the GP IIb/IIIa complex may be involved in uptake of Ca^{2+} by platelets. The complex contains the major part of Ca^{2+} on the platelet surface [73, 77, 221, 302, 592], and Ca^{2+} uptake by platelets is blocked by some antibodies to GP IIb/IIIa [65, 518, 712].

Glycoprotein IV (90 kDa), also known as IIIb, is a major protein which is poorly understood. It is a single chain polypeptide, and it is very hydrophobic [635, 654]. Glycoprotein IV may function as a receptor for thrombospondin [18].

A minor platelet glycoprotein which has received much attention is GP V (82 kDa) [45]. This protein can be hydrolyzed by thrombin [328, 329, 691] and other proteases [46, 411, 701] in a water soluble fragment (69 kDa) and a hydrophobic fragment (20 kDa), left behind in the membrane.

In view of the importance of collagen and thrombin in the physiological stimulation of platelet procoagulant activity, special attention will be given to their mode of action at the surface of the platelet plasma membrane. To initiate platelet aggregation, collagen requires not only its native conformation, a triple helix composed of three polypeptide chains, but also a fibrillar structure [560]. The repeating units dis-

played by collagen fibrils allow for multimeric interactions with collagen receptors, interactions required for optimal platelet response to collagen. Interaction of platelets with collagen can be divided in attachment and spreading. Attachment may involve accessory proteins, which bind both to platelets and collagen, like fibronectin, thrombospondin, and vWf. Studies of a patient with a haemorrhagic disorder, whose platelets lacked GP I_a and were unresponsive to collagen, clearly implicate GP I_a as collagen receptor [455, 456]. These implications have recently been extended by observations that a complex of GP I_a and GP II_a mediates adhesion of platelets to collagen [353, 562], and by a study of another patient with decreased adhesion to collagen [314]. The situation is complicated, however, by suggestions that GP II_b is involved in platelet adhesion to collagen [335, 590, 657]. In addition, specific binding of a 61 kDa [334], a 65 kDa [100], a 80 kDa [359], and a 160 kDa [559] membrane protein to collagen has been described. To reconcile these findings, it seems most likely that platelet stimulation by collagen involves several different binding sites [589]. The interaction of collagen with platelets is reviewed elsewhere [26, 99, 360, 495, 560].

The interaction of thrombin with the platelet plasma membrane is subject to considerable controversy. Despite numerous studies, it is still not clear whether thrombin stimulates platelets by receptor interaction, or by a proteolytic event, or both. Many observations suggest that proteolytic activity of thrombin is required for stimulation of platelets. Inhibition of its proteolytic activity makes thrombin ineffective as platelet stimulator [368], while other proteases, like trypsin, α -clostripain, papain, and thrombocytin, are also able to activate platelets [46]. The best known substrate for thrombin in platelet membranes is GP V [329, 691]. However, despite initial suggestions that GP V hydrolysis may be required for stimulation of platelets by thrombin [410], recent observations clearly suggest that hydrolysis of GP V is not involved in platelet activation by thrombin [56, 300, 409, 691]. In favor for the importance of thrombin receptor interaction are recent observations that small peptides which do not affect the proteolytic activity of thrombin have antagonistic activity [547]. Platelets contain three classes of binding sites for thrombin: a high-affinity, a moderate affinity, and a low affinity

class [270], which recognize different regions of thrombin. The high affinity binding site represents the functional thrombin receptor on platelets [269], though the moderate affinity binding site may take over this function [267]. Early suggestions that GP I_b constitutes a binding site for thrombin, have been confirmed by the effect of antibodies [412, 714], by crosslinking experiments [631], and by direct binding assays [225, 268]. It has been suggested that both the active-site and the fibrinogen binding site of thrombin are involved in binding to GP I_b [260]. However, platelets which lack GP I_b after treatment with selective proteases, still respond to thrombin, albeit retarded [410]. This could be explained by the moderate affinity binding site, which is still present after limited protease treatment, and which may function as thrombin receptor in absence of the high-affinity binding site [267]. In addition, platelet activation by thrombin is not affected by the presence of excess active-site inhibited thrombin, which shows identical binding characteristics as thrombin [46, 328], though inhibition of the proteolytic activity of thrombin can influence its binding to GP I_b [260, 269]. The matter becomes most confusing when Bernard-Soulier platelets are considered. These platelets lack both GP I_b and GP V, yet they respond to high doses of thrombin. To account for these diverse findings, complicated models have been proposed [46], which include proteolysis of the thrombin receptor itself, not necessarily resulting in the loss of a fragment. In addition, it may well be that there is more than one functional for thrombin [409]. Interaction of thrombin with platelets has been discussed in several reviews [43, 46, 160, 199, 495].

Platelet signal transduction

Despite a variety of platelet stimuli (vide supra), platelets display a characteristic sequence of responses upon stimulation. The past ten years ample evidence has been provided that the molecular pathways involved in transduction of the stimulatory signal to the expression of platelet responses are only few in number, and are used by many platelet agonists [reviews: 8, 169, 198, 231, 232, 263, 272, 289, 319, 357, 534, 537, 581, 582, 395a, 605, 646]. A cen-

tral role in platelet activation is played by cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$). This is suggested by observations that Ca^{2+} ionophores are able to elicit the same cellular and molecular responses as physiological agonists do, with the exception of stimulation of phospholipase C [198, 538]. The use of permeabilised platelets [581, 582] further demonstrated the importance of $[\text{Ca}^{2+}]_i$ and the effects of nucleotides and protein phosphorylation on the Ca^{2+} requirements for platelet activation. In addition, the use of intracellular fluorescent (chlorotetracycline, Quin2, Fura2) and luminescent (aequorin) calcium indicators provided detailed information about $[\text{Ca}^{2+}]_i$ in terms of sources, levels, and regulation [191, 263, 264, 265, 303, 434, 508, 510, 525, 532, 534, 535, 550, 641, 680, 681]. Resting $[\text{Ca}^{2+}]_i$ in platelets is around 80–100 nM [508, 521, 535]. During platelet activation $[\text{Ca}^{2+}]_i$ will rise to 1–5 mM due to mobilization of intracellular Ca^{2+} and influx of extracellular Ca^{2+} [169, 263]. In addition to $[\text{Ca}^{2+}]_i$, and in synergistic cooperation, protein kinase C is clearly involved in platelet signal transduction [231, 305, 462]. More recently, guanine nucleotide-binding regulatory proteins (G-proteins) have entered the scheme as a direct link between receptors and phospholipases and ion-channels [81, 272, 386, 452]. Though understanding of the mechanisms of signal transduction in platelets has gained much, in-

volvement of as yet unidentified mechanisms may operate in platelets activated by ADP [534]. Platelet activation is complicated by the actions of autocoids, especially TxA_2 [8, 357], formed during platelet stimulation and functioning as a positive feedback. Finally, inhibition of platelet activation, via the production of cAMP, is provided by prostacyclin, resulting from the stimulation of endothelial cells by ADP and thrombin, and partly dependent on platelet derived endoperoxides [357, 646].

It has become evident that one of the first actions of stimulatory agonists on platelets is a rapid degradation of polyphosphoinositides (phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIP₂)) by polyphosphoinositide-specific phospholipase C (a phosphodiesterase), resulting in the formation of inositol-1,4,5-trisphosphate (IP₃) and 1,2-diacylglycerol (DAG) [145, 169, 272, 706]. The degradation and resynthesis of phosphoinositides (PI-cycle) has evolved into a complex set of reactions (Fig. 4). The DAG formed is enriched with stearate and arachidonate, and for a large part rapidly phosphorylated to stearyl arachidonyl phosphatidic acid (PA) [407], often used as an indicator for phospholipase C activity. In thrombin-activated platelets, the cellular content of PIP₂ is rapidly decreased followed by a compensatory resynthesis above resting values, while the content of PIP is

Fig. 4. Metabolism of phosphoinositides and inositol phosphates in platelets, and the importance of some of its components in platelet activation. Abbreviations are explained throughout the text. All inositols and inositides have the D-myo-inositol configuration. Figure is taken from [272].

not altered. Resynthesis of polyphosphoinositides results in a drop in the content of PI. The action of phospholipase C results in the generation of various inositol 1:2-cyclic phosphates [297, 638] and inositol 1-phosphates. Best known is IP₃, which is dephosphorylated back to inositol, or phosphorylated to I(1,3,4,5)P₄ (IP₄) and even higher phosphates. cl(1:2,4,5)P₃ is hydrolysed much more slowly than IP₃ and was found to release Ca²⁺ from permeabilized platelets with a similar potency as IP₃ [705]. It has become clear that the stimulation of phospholipase C by agonist-receptor complexes is mediated by G-proteins [272, 366, 367]. G-proteins are membrane-bound heterotrimers, consisting of an α , β , and γ -subunit, of which the α -subunit contains the GTP binding-site. G-proteins become active upon receptor occupation by exchanging bound GDP for GTP, and are inactivated by an intrinsic GTPase activity. The G-protein that stimulates phospholipase C is different from those which stimulate (G_s) or inhibit (G_i) adenylate cyclase, and is referred to as G_p [111]. Phospholipase C can be inhibited by cyclic AMP (cAMP), most likely by increasing the GTPase activity of G_p [272]. The second messengers generated by phospholipase C are DAG, IP₃, and probably IP₄. DAG will stimulate protein kinase C (C-kinase) [460, 461], IP₃ is able to promote Ca²⁺ release from the DTS [6, 75, 169, 478, 527, 588], and IP₄ is suggested to be involved in influx of extracellular Ca²⁺ [293].

C-kinase consists of a family of enzymes that transfer phosphate from ATP to serine or threonine residues of specific proteins in the presence of PS and Ca²⁺ [459]. DAG stimulates C-kinase by lowering its Ca²⁺ requirement to submicromolar levels. C-kinase can be stimulated by some phorbol esters and synthetic diacylglycerols. An alternative way to stimulate C-kinase is suggested by observations that limited proteolysis by calpain renders C-kinase active independent of PS, DAG and Ca²⁺ [323, 636]. An important aspect in stimulation of C-kinase is its translocation from the cytosol in resting platelets to the membrane in response to cell stimulation. Mechanisms governing the translocation of C-kinase are poorly understood, and may involve increased Ca²⁺ concentrations. C-kinase can modulate both stimulatory and inhibitory pathways of platelet activation, and is thus suggested to be a bidirectional cell modulator [169]. The stimulatory ac-

tions of C-kinase operate in harmony with the actions of Ca²⁺ to produce synergistic responses [462, 632]. The major substrate of C-kinase in platelets is a 47 kDa protein (P47). P47 is a soluble protein, and its phosphorylation is extensively correlated with the release reaction, more specifically with granule fusion [231]. The identity of P47 is not known, though several hypotheses have been advanced [272]. Phosphorylation of filamin by C-kinase during platelet activation has been postulated to regulate the cytoskeletal reorganization involved in the formation of pseudopodia [231]. An other group of important substrates of C-kinase are the α -subunits of various, if not all, G-proteins [81, 169, 272, 386, 452]. By phosphorylation of G_i- α , C-kinase suppresses the stimulation of adenylate cyclase. Inhibition of phospholipase C, evidenced by the effects of phorbol ester on PIP₂ hydrolysis and mobilization of intracellular Ca²⁺, is most likely due to phosphorylation of G_p- α . Inhibition of influx of extracellular Ca²⁺ through ion-channels by phorbol ester [504, 505] can also be explained by the action of C-kinase towards G-proteins. Phosphorylation of MLC by C-kinase constitutes another inhibitory action, resulting in suppression of actin-activated ATPase activity of myosin which has been phosphorylated by MLC kinase [231].

Cytosolic Ca²⁺ increases during platelet activation as a result of mobilisation of Ca²⁺ from the DTS by IP₃, and influx of extracellular Ca²⁺ through receptor-operated divalent ion-channels (ROC's) [169, 263, 272, 533, 581, 646]. In case of threshold agonist concentrations, Ca²⁺ mobilization is dependent on cytosolic alkalization by the Na⁺/H⁺ exchanger [597]. Important pathways by which increased [Ca²⁺]_i exerts its intracellular effects are: Ca²⁺/calmodulin-dependent processes, Ca²⁺-dependent neutral cysteine protease (calpain), and phospholipase A₂. In addition, Ca²⁺ directly affects the cytoskeletal organization by modulation of the binding properties of cytoskeletal proteins and by microtubule depolymerization [316, 427], and Ca²⁺ facilitates fusion between granule membranes and the plasma membrane [385]. Calmodulin can bind up to 4 Ca²⁺ ions, leading to substantial conformational changes. The Ca²⁺/CaM complex modulates the activity MLC kinase [231], and phosphorylase b kinase [646] which regulates glycogenolysis. In addition, CaM has been shown in other cells to be

involved in Ca^{2+} -dependent regulation of cyclic nucleotide phosphodiesterase, Ca^{2+} -dependent protein phosphatase (calcineurin), adenylate cyclase, and the calcium pump ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase) [70, 135, 178, 646]. In platelets, adenylate cyclase and cyclic nucleotide phosphodiesterase may not be regulated by CaM [692]. Regulation of cytoskeletal organization by Ca^{2+} /CaM is thought to proceed via a so-called "flipflop" mechanism [465], i.e. caldesmon binds to actin at low $[\text{Ca}^{2+}]_i$ but binds to CaM at elevated $[\text{Ca}^{2+}]_i$. This switch in binding of caldesmon is believed to act as an on-off switch for actin-myosin interaction. To which extent CaM exerts the same effects in platelets is not clear [646]. Limited proteolysis by calpain may be an underestimated mediator of the Ca^{2+} signal. Among its substrates are many cytoskeletal and membrane-bound proteins. Of particular interest for platelet signal transduction are the findings that C-kinase [323, 636], phospholipase C [390], MLC kinase [306, 307, 675], and adenylate cyclase [4, 647] can be irreversibly activated by calpain. In view of its importance in the context of this thesis, calpain will be discussed in more detail elsewhere (vide infra).

Of special importance in mediating the Ca^{2+} signal in platelets is the hydrolysis of phospholipids by phospholipase A₂ [272, 537]. Evidence is increasing that phospholipase A₂ exists in multiple forms with different Ca^{2+} requirements [22]. This can explain the paradoxical findings that platelet phospholipase A₂ can be stimulated by increased $[\text{Ca}^{2+}]_i$ [97, 337, 508], but also independent of $[\text{Ca}^{2+}]_i$ by receptor occupation [83, 272, 508]. Part of phospholipase A₂ is bound to the plasma membrane and the DTS [138], while the majority is soluble [337]. It seems most likely that phospholipase A₂ bound to the plasma membrane is activated directly by a G-protein after agonist-receptor binding [83, 223, 443, 444, 445], and that a soluble phospholipase A₂ becomes bound to membranes only after Ca^{2+} mobilization. The important product generated by phospholipase A₂ is arachidonic acid, which is derived from PE, PC, and PI [78, 605]. Arachidonic acid can also be formed from DAG, produced by phospholipase C, via the action of a di- and mono-acylglycerol lipase [272, 357, 537]. Arachidonic acid is a substrate for two enzymes: cyclooxygenase and lipoxygenase [319, 357, 537]. Cyclooxygenase converts arachidonate to

prostaglandin endoperoxide G₂ (PGG₂), which is further reduced to PGH₂. The endoperoxides PGG₂ and PGH₂ are unstable proaggregatory molecules. PGH₂ is further metabolized by thromboxane synthase to TxA₂, a powerful stimulatory platelet agonist. TxA₂ is rapidly inactivated by hydrolysis to TxB₂. Cyclooxygenase is irreversibly inhibited by aspirin. Lipoxygenase converts arachidonate to 12HPETE (12-hydroperoxy-5,8,10,14-eicosatetraenoic acid), which is reduced to 12HETE (12-hydroxy-5,8,10,14-eicosatetraenoic acid). Several reports suggest that lipoxygenase products may oppose the aggregating activity of endoperoxides and TxA₂ [357].

Consistent with a central role of $[\text{Ca}^{2+}]_i$ in platelet signal transduction, the regulation of Ca^{2+} , in unstimulated platelets as well as in stimulated platelets, is intricately controlled by homeostatic mechanisms [74, 89, 169, 376]. Cytosolic Ca^{2+} homeostasis is maintained by concerted operation of transport systems located in mitochondria, endoplasmic reticulum, and plasma membrane. Mitochondrial Ca^{2+} homeostasis is regulated by two transport systems: an energy-dependent uniport carrier and a less well-defined release mechanism involving a $\text{H}^+/\text{Ca}^{2+}$ exchange [87, 531]. Mitochondrial Ca^{2+} pool is depleted upon oxidation of NAD(P)H. Active transport of calcium ions into the DTS and to a lesser extent over the plasma membrane is achieved by two distinct $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPases [185, 188, 254]. The $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPases from the DTS is subject to bidirectional modulation by cAMP-dependent phosphorylation and CaM/ Ca^{2+} -dependent phosphorylation [5, 376, 719]. In view of the involvement of free thiol groups in Ca^{2+} -ATPase function [580], maintenance of the cellular redox status may be important for optimal $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity. Transport over the plasma membrane can also be achieved by a $\text{Na}^+/\text{Ca}^{2+}$ exchanger [528]. In activated platelets, additional Ca^{2+} transport systems are evoked: Ca^{2+} channels in the plasma membrane (ROC's) and the DTS. Influx of Ca^{2+} via ROC's is under negative feedback control of C-kinase [169, 395, 533] and probably $[\text{Ca}^{2+}]_i$, and may be modulated by G-proteins [81, 244]. Mobilization of Ca^{2+} from the DTS by IP₃ is dampened by $[\text{Ca}^{2+}]_i$ and suppressed by cAMP-dependent inhibition of phospholipase C [263, 395, 581].

In platelets, stimulatory pathways are antagonized by cAMP-dependent processes [581, 646]. cAMP is produced from ATP by adenylyl cyclase, which is exclusively located at the cytosolic side of the platelet plasma membrane [418]. Adenylyl cyclase activity is modulated by an inhibitory (G_i) and stimulatory (G_s) G-protein [235, 236, 381]. Inhibitory platelet agonists (PGE₁, PGI₂, and adenosine) stimulate adenylyl cyclase. Excitatory agonists (thrombin, collagen, PAF) and proaggregatory agonists (ADP, epinephrine) inhibit adenylyl cyclase [646]. The propagation of the inhibitory signal is ensued by cAMP-dependent kinase (A-kinase). Platelets contain two A-kinases: a membrane bound type I, and a soluble type II [231]. Important substrates of A-kinase are cAMP phosphodiesterase, filamin, and MLC kinase [231, 646]. cAMP phosphodiesterase actually is under bidirectional control: stimulation by A-kinase provides an important negative feedback, which is abolished by increased $[Ca^{2+}]_i$. Phosphorylation of MLC kinase by A-kinase results in substantial inhibition of its ac-

tivity towards MLC. Other substrates of A-kinase are two proteins with a molecular weight of 22 kDa (P22) and 24 kDa (P24). The function of these proteins and their cAMP-dependent phosphorylation is not clear at present. In addition to phosphorylation, stimulation of adenylyl cyclase results also in dephosphorylation of MLC (P20) and P47, which is associated with disaggregation and cytoskeletal disassembly, and which may result from inhibition of phosphorylation by MLC kinase and C-kinase, or from stimulation of cAMP-dependent phosphatases. The most obvious effect of stimulation of adenylyl cyclase is the inhibition of Ca^{2+} mobilization, both influx from extracellular Ca^{2+} and release from intracellular stores. The latter probably is a result of inhibition of phospholipase C by increased cAMP levels [581]. An intriguing possibility arises regarding recent developments in G-protein research [81, 272, 386, 452]. In view of newly discovered GTP-binding proteins in platelets [55, 193] in the range of 21-27 kDa, and in view of the regulation of G_α subunits by phosphorylation, it may

Fig. 5. Schematic representation of the platelet signal transduction system. For sake of clarity, only the production of second messengers is described. Inhibitory and stimulatory interactions between different systems and their target proteins are described throughout the text. As a general rule signal transduction starts with receptor (R) activation by agonistic ligands (L), and is mediated at the cytosolic side of the plasma membrane (PM) by G-proteins which stimulate key-enzymes to produce second messenger molecules. Stimulatory pathways involve phospholipase C (PL-C), phospholipase A₂ (PL-A₂) and receptor operated Ca^{2+} channels (ROC's). The inhibitory pathway involves adenylyl cyclase (AC). Further abbreviations are defined in the text. Stimulation of ROC's and phospholipase A₂ by G-proteins is still speculative, as well as the topography of the phospholipase A₂ system.

well be that cAMP-dependent phosphorylation of P22 and P24 is linked to inhibition of plasma membrane Ca^{2+} channels (ROC's) and phospholipase C. Inhibition of Ca^{2+} release from the DTS may also result from cAMP dependent inhibition of IP_3 induced Ca^{2+} release [187].

The function and significance of cGMP in platelets is unclear [646]. Platelets contain guanylate cyclase, a cGMP phosphodiesterase, but no cGMP-dependent kinase. Platelet guanylate cyclase is a soluble heme protein, which is de-

pendent on the redox-status of the cell, and which can be activated by stimulatory agonists via a Ca^{2+} -dependent step, and by polyunsaturated fatty acids. The cGMP phosphodiesterase is stimulated by PGE_1 , suggesting control by inhibitory agonists. Interaction between the cAMP and cGMP system occurs mainly at the level of hydrolysis of each others cyclic nucleotides.

To reconcile platelet signal transduction, the main pathways are modeled in Fig. 5.

CALPAIN

Characteristics

Calpain is an ubiquitous proteolytic enzyme (E. C. 3. 4. 22. 17), dependent on Ca^{2+} and with a thiol group in the active centre requiring neutral pH for its activity [433]. Based on its Ca^{2+} -dependence, two subclasses of calpain are recognized; calpain-I and calpain-II, which show halfmaximal activity at 20-50 μM and 0. 2-0. 5 mM Ca^{2+} , respectively. Calpain is a heterodimer consisting of a small and large subunit [624, 652]. The small subunit of 30 kDa is similar for both calpain-I and calpain-II [179, 687], and functions as a regulatory subunit [179, 295, 312]. The large subunit (about 80 kDa), is different for calpain-I and calpain-II [180, 687] and contains the active center [652]. Based on its amino-acid sequence, it is suggested that the large subunit arose from a gene fusion of calmodulin and a thiol protease [181]. Calpain is dependent on a reduced thiol group for full activity [625], and can be inhibited by SH-modifying agents (diamide, NEM, mersalyl acid) or by more specific oligopeptide-inhibitors like leupeptin and antipain. Calpain and its properties have been reviewed elsewhere [393, 394, 431, 432, 433, 511, 514, 573, 622, 624, 627, 682].

Regulation

It has long been thought that the relatively high Ca^{2+} requirement of calpain would ex-

clude a role for calpain in intracellular mechanisms. Interest in calpain, however, has clearly revived considering the vast amount of literature concerned with various aspects of cellular calpain. This interest in calpain is probably related to the manifold manipulations of calpain activity occurring within cells, which suggest that calpain can be subject to fine-tuning of its activity, making a role in cellular function most likely.

Autoactivation has been suggested to lower the Ca^{2+} -dependence of calpain-II, by removal of a small fragment from the heavy subunit [130, 276, 626, 628, 629]. As a result, an additional binding site with a much higher affinity for Ca^{2+} would be uncovered. The Ca^{2+} requirement for autoactivation of calpain-II can be reduced by binding to PI containing membranes [129, 295]. Autoactivation of calpain-I has also been reported [517], and is dependent on binding to the cytosolic side of the plasma membrane [346]. In this case an inactive pro-enzyme with a molecular weight of several hundred kDa is transformed into calpain-I, without any change in affinity for Ca^{2+} .

Just as the positive feedback via autoactivation, calpain is also subject to negative feedback via autolysis, rendering calpain inactive [296]. This property is shared by both calpain-I and -II, and may be an important way to terminate the action of calpain thereby allowing spatially and temporally localized protein degradation by calpain.

Calpastatin is an endogenous inhibitor of calpain found in all cells where calpain is pres-

ent [431, 432, 433]. Calpastatin can bind to membranes [416]. The molecular weight differs greatly among various sources, which may be caused by different degrees of polymerization. Regulation of calpain by calpastatin may turn out to be very complex. Calpastatin has been found to keep inactive calpain independent of Ca^{2+} [133], or to inhibit calpain after binding of Ca^{2+} [134]. Calpastatin can inhibit binding of calpain to membranes [517]. In addition, calpastatin itself is a substrate for calpain [417]. Calpastatin does not discriminate between calpain-I and -II [431].

The major determinant of the relevance of calpastatin in a particular cell may be the quantitative balance between calpain and calpastatin [431, 432, 433]. In erythrocytes, which contain calpastatin in excess of calpain-I [241], treatment with a Ca^{2+} ionophore in the presence of millimolar Ca^{2+} does only lead to cytoskeletal degradation after prolonged incubations [95, 248, 389], or after addition of purified calpain [140]. In contrast, calpain can easily be activated by ionophore treatment in platelets [124, 664, 690], which contain calpain-I in excess of calpastatin [431, 432, 433].

Binding of calpain to membranes is facilitated by the small subunit [295]. This subunit contains a high amount of hydrophobic amino acids at the N-terminus, while the C-terminus represents a Ca^{2+} binding region [179], suggesting that membrane binding can be regulated by Ca^{2+} . Binding of calpain to membranes has been reported to be dependent on anionic phospholipids like PS and PI [129]. In resting cells, calpain is present in a soluble form in the cytosol. This would mean that during cell activation translocation of calpain to the plasma membrane may occur. Membrane binding of calpain may be an important way to limit the action of calpain to membrane surfaces [415], or to localize calpain near its substrates.

Calpain can be phosphorylated *in vitro* by protein kinase C [283]. Phosphorylation only involves inactive calpain, suggesting a regulatory role for phosphorylation. *In vivo*, phosphorylation of calpain could not be demonstrated [1]. Thus, the importance of phosphorylation in regulation of calpain activity is at present unclear.

In Triton-insoluble residues from bovine brain [156, 633], platelets [633], and neutrophils [515], a 20-40 kDa activator of calpain has been found. This activator increased calpain activity

without changing its sensitivity for Ca^{2+} . The relevance of this small protein for regulation of calpain activity is still unclear. The effect of the activator on calpain can be antagonized by calpastatin [515], which suggests that calpain may be modulated *in vivo* by dynamic interaction between activator and inhibitor.

In principle, enzyme activity can be dependent on the structure of its substrate. Evidence is accumulating that phosphorylation of substrate proteins modulates their susceptibility towards calpain. Phosphorylation of filamin prevents degradation by calpain [723]. Phosphorylation of cytoskeletal proteins by C-kinase in neutrophils [512] and cAMP-dependent phosphorylation of troponin [645] triggers their degradation by calpain. Substrate modulation may be a common way of restricting calpain activity to particular pools of substrates. The main substrates of calpain (*vide infra*) are subject to structural changes upon cell activation.

Substrates

Substrates of calpain fall in two main categories: cytoskeletal proteins and membrane proteins. In fact, a common denominator of both categories may be the binding of calpain to membranes. In general, proteolysis by calpain is characterized by its limited action, normally producing only few fragments. The substrate specificities of calpain-I and calpain-II are quite alike but not identical. Both calpains prefer an Arg or Lys preceded by a Leu or Val [431].

Cytoskeletal substrates of calpain cover the whole range of cytoskeletal structures: microtubules, intermediate filaments, and actin filaments. Both tubulin [58] and microtubule-associated proteins [58, 563] can be degraded by calpain. Intermediate filaments (vimentin, desmin) [453, 493, 570] and neurofilament proteins [464, 485, 672] are documented substrates for calpain. Many actin-binding proteins can be hydrolysed by calpain: spectrin [215, 296], ankyrin [262], band-4.1 [140], fodrin [393, 463, 672], filamin [212], talin [30, 31, 122, 212, 470, 690], myosin [296], α -actinin [717], caldesmon [675], tropomyosin [682], and vinculin [280]. Most peculiar, actin is not degraded by calpain

[679], suggesting that the action of calpain is not meant to destroy the cytoskeleton but restricted to change its organization. Limited hydrolysis of the high-molecular weight actin-binding proteins results mostly in cleavage of a small fragment. In case of filamin, the large fragment is still able to bind actin [93], but has lost its capacity to crosslink actin filaments [150, 151, 648], again suggesting that limited proteolysis of cytoskeletal proteins must lead to reorganization but not destruction of the cytoskeleton. This is further documented by the observation that the interaction of filamin with the actomyosin ATPase [143, 475, 610] is abolished after limited proteolysis by calpain [474]. In some cells [464, 664, 690] millimolar Ca^{2+} calpain is able to degrade cytoskeletal proteins to many small fragments in the presence of millimolar Ca^{2+} .

Membrane-bound substrates of calpain can be further divided in two classes: transmembrane receptors and cytosolic proteins. Limited proteolysis by calpain can modulate several receptors: epidermal growth factor receptor [227, 251], platelet derived growth factor receptor [177], glutamate receptor [599], and insulin receptor [375]. As a result, receptor properties will be changed, like autophosphorylation, coupling to cytoskeletal proteins or ligand-binding characteristics. Calpain can also affect several key proteins in signal transduction and cellular Ca^{2+} homeostasis. Adenylate cyclase [4, 647], C-kinase [252, 283, 323, 414, 512, 513, 515, 516, 565, 637], A-kinase [553], phospholipase C [390], CaM-dependent phosphatase [675], MLC kinase [306, 307, 675], and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase [20, 678] become irreversibly activated upon limited proteolysis by calpain.

A number of extracellular substrates have been described. Among these are the platelet glycoproteins I_b [411, 713] and V [56, 411], fibrinogen [351], von Willebrand factor [352], and high molecular weight kininogen [571]. In addition, several proteins involved in coagulation can be hydrolysed by calpain: factor V [69], factor XIII [11], and protein S [421]. However, in intact cells calpain is confined to the cytosol [431, 555], suggesting that extracellular substrates are only relevant in case of cell damage.

Function

Stimulation of calpain leads to irreversible changes in protein structure and function. Thus, any function of calpain in cellular processes is likely to be dependent on this irreversibility, and would involve long-term changes. Although the function of calpain in cellular processes is only beginning to be understood, some examples have been investigated in more detail. In brain, calpain-I mediated degradation of fodrin (brain spectrin) increases irreversibly the number of functional glutamate receptors, thereby providing a plausible mechanism for long-term memory [393, 394]. In chicken erythropoiesis, calpain is involved in the construction of the membrane skeleton, by eliminating unused cytoskeletal proteins tagged by post-translational modification [372, 708]. In erythrocytes [7, 241, 242] and myoblasts [574, 575], calpain has been suggested to play a role in membrane fusion by clearing part of the membrane from the underlying network of cytoskeletal proteins. Calpain may be involved in cellular destruction, considering the massive proteolysis during myocardial ischemia [24, 255, 277, 374, 644, 651], and myelopathy [23]. Finally, calpain may be involved in the regulation of the microtubular reorganization during mitosis [252, 573]. Obviously, the functions described are in accordance with the substrate specificity of calpain: cytoskeletal proteins apparently are the primary targets. The listed functions, however, lack examples of involvement of calpain in signal transduction, despite ample indications that such an involvement would be feasible (vide supra).

Calpain in platelets

In platelets, calpain has been subject of extensive research since its discovery in 1977 [499]. Platelet calpain mainly consists of calpain-I [212, 333, 552, 655, 679], and is a cytosolic protein [555] responsible for almost all Ca^{2+} -dependent proteolytic activity in platelets [411, 499, 648]. Calpastatin is present in platelets only in minor amounts [431, 433, 630]. A low molecular weight activator of calpain has been found in platelets [633].

Platelet calpain activity can easily be monitored by the degradation of its major substrates and the formation of their fragments as judged from protein patterns after gel electrophoresis of reduced protein samples (Fig. 6). The major substrates of calpain are the cytoskeletal proteins filamin, talin, and myosin [124, 216, 499, 608, 656, 690]. Their major fragments are found at 190 kDa (fragment 1), 135 kDa (fragment 2), 93 kDa (fragment 3), and at 48 kDa (fragment 4) [119, 124, 216, 442, 499, 608, 690]. Filamin is degraded by calpain into fragment 1 and fragment 3 [212, 648]. Fragment 2 most likely represents the myosin rod cleaved from heavy chain myosin [296, 492]. Talin is degraded by calpain to a protein band corresponding with fragment 1, indistinguishable from that of filamin, and to fragment 4 [31, 122, 212, 292, 470]. Platelet α -actinin can also be hydrolysed by calpain [522, 717].

Degradation of filamin results in a decoupling of glycoprotein Ib from the membrane skeleton [206, 207, 472, 608], and a loss of the actin-gelating capacity of filamin [150, 151,

648]. Decoupling of glycoprotein Ib from the membrane skeleton results in a reorganization of this glycoprotein in the plasma membrane, and changes in its receptor properties. Degradation of talin may be related to a reduction in high affinity binding sites for fibrinogen on the glycoprotein IIb/IIIa complex [490]. Several proteins involved in signal transduction can become irreversibly activated by calpain: adenylyl cyclase [4, 647], C-kinase [323, 636], phospholipase C [390], and MLC kinase [306, 307, 675].

Limited calpain activity has been observed in thrombin-activated platelets, only under stirring conditions and several minutes after addition of thrombin [208, 212]. It has been suggested that this limited degradation of cytoskeletal proteins is a prerequisite for the formation of a contractile skeleton. Stimulation of platelets by the Ca^{2+} ionophore does result in massive degradation of cytoskeletal proteins [124, 664, 690], indicative of the potency of platelet calpain.

REGULATION OF TRANSBILAYER LIPID ASYMMETRY

Transbilayer movement in lipid model systems

A lipid bilayer is generally a stable structure held together due to thermodynamically and hydrodynamically favoured interaction between amphipathic lipids [164, 298, 549]. Transbilayer movement of phospholipids (flipflop) would require the charged and hydrated headgroup to pass the hydrophobic bilayer interior. This process is characterized by a high activation energy. Thus, flipflop in stable unilamellar vesicles is a relatively slow process, occurring at time scales of days to weeks. Flipflop can be considerably enhanced by introduction of local defects in lipid bilayer packing [154].

Introduction of different physical properties between the two monolayers of a lipid membrane has been shown to induce enhanced

rates of flipflop (fliprates). Differences in physical properties between monolayers include differences in headgroup composition [339], differences in phase behavior [344], and differences in fluidity [343]. Difference in headgroup composition refers to an asymmetric distribution of phospholipids, i.e. a concentration gradient over the membrane. Such a concentration gradient increases the free energy of lipids, and will allow more lipids to pass the threshold of activation energy for flipflop, resulting in higher fliprates. Differences in headgroup composition between the two monolayers of a membrane may also lead to formation of lipid bilayer instabilities (vide infra). Induction of a phase-separation in unilamellar vesicles of PC results in enhanced fliprates, as a result of packing defects at the border of gel-liquid crystalline phase transition [344]. Differences in physical properties between both monolayers

of a membrane may find a biological correlate in the transbilayer fluidity gradient, which has been observed in most cells in which it was investigated [318, 429, 587].

Insertion of transmembrane proteins may result in increased fliprates [233, 612, 726]. Incorporation in artificial vesicles of glycophorin and band-3, membrane proteins isolated from the erythrocyte, substantially increases the fliprate of lyso-PC [726]. This is most likely due to the inability of a single phospholipid species to accommodate the irregularities at the interface of the membrane spanning proteins and the lipid bilayer, since more complex lipid mixtures, like total erythrocyte lipids, do not exhibit enhanced fliprates upon incorporation of glycoprotein [233]. Most interesting is the observation that clustering of glycoprotein in PS/PE vesicles by wheat germ agglutinin results in destabilization of the lipid bilayer and hence increased fliprates of phospholipids [612]. Ligand-dependent clustering of membrane glycoproteins is a common phenomenon in cells, and may thus constitute a biological mechanism to increase the fliprate in plasma membranes.

A subject of extensive research is the formation of non-bilayer structures in model as well as in biological membranes [141, 341]. Formation of lipidic structures is related to

their molecular shape. Cylindrical phospholipids like PC and SphM will form lipid bilayers upon hydration. Lipids like PA, PE with unsaturated acyl chains, and diacylglycerides have a cone-like shape, and will adopt hexagonal structures upon hydration, such as tubes and inverted micelles with the acyl chains pointing outward. These lipids are abundant components of biological membranes, and have been implicated in membrane fusion and flipflop. Generally, the presence of non-bilayer lipids in membranes does not necessarily result in disturbances of the bilayer structure [234, 642]. This is due to the presence of other lipid species counteracting the formation of non-bilayer structures. However, if the lipids become heterogeneously distributed in the membrane, domains of non-bilayer lipids may form and non-bilayer structures will occur [234, 642]. In this way, formation of non-bilayer structures can be under control of potentially physiological mechanisms, like clustering of PS by increased Ca^{2+} levels [234, 642], or clustering of transmembrane proteins [612].

The relevance of non-bilayer lipidic structures, such as inverted micelles, for enhanced fliprates can be questioned, because these structures apparently represent a thermodynamically favoured situation. However, since the fraction of phospholipids participating in fast isotropic motion, as observed by ^{31}P NMR, is much larger than the fraction participating in non-bilayer structures, as observed by freeze fracture electron microscopy, it has been argued that rapid exchange between bilayer and non-bilayer lipid occurs [342], thereby providing means for rapid transbilayer movement of phospholipids. Involvement of non-bilayer structures in rapid flipflop is further suggested by their relation in model systems [172], as well as biological membranes [171], though it may well be that non-bilayer structures are not necessarily involved in rapid transbilayer movement of phospholipids in biological membranes [107]. Formation of non-bilayer structures in biological membranes may be facilitated by the composition and distribution of the phospholipids. Concentration of PE with a large amount of polyunsaturated acyl chains at the cytosolic side of the plasma membrane will favour formation of non-bilayer structures under certain conditions, like increased Ca^{2+} levels [291].

Fig. 6. Degradation of platelet cytoskeletal proteins by calpain. Numbered arrows indicate characteristic fragments, which are described in the text. Protein patterns were obtained before and after stimulation of human platelets by the Ca^{2+} ionophore A23187 in the presence of millimolar extracellular Ca^{2+} . For technical details is referred to chapter 2.

Transbilayer movement in biological membranes

Regulation of transbilayer lipid asymmetry in biological membranes has been subject of several reviews [476, 477, 479, 545, 577], and is best studied in the erythrocyte. Erythrocytes have only one membrane, the plasma membrane. The phospholipids are asymmetrically distributed over the plasma membrane, quite similar as in platelets: choline-phospholipids and amino-phospholipids are enriched in the outer and inner leaflet, respectively [404, 666, 736]. The lifespan of erythrocytes, about 120 days, is much longer than that for platelets. This long lifespan has led to numerous investigations of the mechanisms by which transbilayer lipid asymmetry is maintained. Originally these investigations focussed on the role of the membrane skeleton [16, 39, 104, 168, 217, 256, 349, 423, 424, 572, 703, 704]. More recently, however, interest has changed focus on the role of a phospholipid transport protein [127, 144, 281, 406, 585, 643, 702, 721].

The cytoskeleton of erythrocytes covers the cytosolic face of the plasma membrane, and was the first example of a membrane skeleton. The membrane skeleton is a network of spectrin and actin, which is coupled to the plasma membrane by binding to integral membrane proteins [reviewed in: 36, 37, 71, 114, 245, 256, 392, 401, 422, 483, 484, 595, 669, 704, 716]. The suggestion that the membrane skeleton could be involved in the maintenance of transbilayer lipid asymmetry, came from experiments which demonstrated an interaction between spectrin and lipids of the cytosolic leaflet of the plasma membrane [425, 426], and experiments in which crosslinking of spectrin resulted in loss of transbilayer lipid asymmetry [257, 259]. Structural modifications of the membrane skeleton, either *in vitro* or in pathological erythrocytes, further confirmed the notion that anionic phospholipids were held at the cytosolic leaflet of the plasma membrane by direct interaction with spectrin [16, 39, 104, 168, 217, 256, 349, 423, 424, 572, 703, 704]. Direct interaction between erythrocyte cytoskeletal proteins and membrane lipids has been frequently observed by now, not only for spectrin [64, 112, 398, 425, 426, 674] but also for band 4.1 [113, 564, 596, 674], a protein that links the membrane skeleton to the erythrocyte plasma membrane. In

general, interaction between cytoskeletal proteins and the lipid bilayer is dependent on electrostatic attraction, followed by consolidation of binding by additional hydrophobic interactions.

Structural disturbance of the erythrocyte membrane skeleton may not necessarily result in net transbilayer movement of PS, but it generally leads to enhanced rates of transbilayer movement. Thus it is observed that exogenously added lyso-PC or endogenous PC become subject to increased fliprates whenever the structure of the membrane skeleton is damaged [218, 219, 220, 258, 354, 477]. Apparently, the structural integrity of the lipid bilayer is dependent on an intact membrane skeleton. This suggestion is supported by results from direct measurements of membrane structure by lipidic probes [15, 95, 103, 104, 106, 194, 408, 526, 703].

Application of various phospholipid probes to erythrocytes clearly demonstrated the occurrence of rapid transbilayer movement from the outer to the inner leaflet of the plasma membrane, specific for PS, and to a lesser extent for PE [144, 406, 585, 643, 721]. This transbilayer movement is dependent on intracellular ATP levels, and is characterized by a halftime of approximately 7 minutes for PS, which is much higher than passive rates of transbilayer movement through lipid bilayers. The phospholipid transport activity is inhibited by thiol oxidizing agents, which lead to the suggestion that a protein was responsible for the observed ATP-dependent transport of PS over the plasma membrane. This putative protein has been named aminophospholipid specific translocase [721], and its presence has been demonstrated in various cell types [406, 722], including platelets [623]. The translocase is inhibited by intracellular Ca^{2+} levels above $1 \mu\text{M}$ [721], and stereospecifically interacts with amino-phospholipids [406].

As to the relative contribution of cytoskeleton and translocase in the maintenance of transbilayer lipid asymmetry, consensus is not reached. It has been argued that the transbilayer distribution of PS and PE can solely be explained by the ratio between inward and outward rate-constants of the translocase [281], while others argue that a translocase with identical outward and inward rate-constants in cooperation with selective binding of aminophospholipids to the membrane skeleton are responsible for the observed phospholipids

transbilayer asymmetry [702]. It seems most likely, considering the ample evidence for direct interactions between membrane lipids and cytoskeletal proteins, that the membrane skeleton is involved in the maintenance of phospholipid transbilayer asymmetry, e. g. by maintaining the structural properties of the lipid bilayer.

With respect to the loss of transbilayer lipid asymmetry, the erythrocyte does not give definite clues. Original findings that increased flippases after manipulation of cytoskeletal organization did not result in exposure of PS, were taken as evidence for selective interaction of PS with cytoskeletal proteins [vide supra]. This conclusion may have been premature, since an alternative explanation would be the selective action of a translocase. Recent experi-

ments in which both cytoskeletal structure and translocase function were individually manipulated [420], however, seem to support the original assumption that cytoskeletal proteins contribute to the maintenance of phospholipid transbilayer asymmetry by binding anionic phospholipids at the cytosolic leaflet of the plasma membrane.

With respect to the loss of transbilayer lipid asymmetry during platelet activation, the most relevant observation probably is that a decreased interaction between cytoskeletal proteins and the plasma membrane increases the rate of transbilayer movement over the plasma membrane [vide supra], resulting in a loss of transbilayer lipid asymmetry in the absence of any other regulatory factor.

PLATELET PROCOAGULANT ACTIVITY

Introduction

The interaction of various coagulation factors with activated platelets plays an essential role in hemostatic plug formation. In particular, the platelet surface membrane interacts with factors X_a and V_a to ensure formation of the prothrombinase complex, as well as promoting the assembly of the factor IX_a - $VIII_a$ complex required for activation of factor X. Binding to activated platelets dramatically enhances the enzymatic activity of these complexes of coagulation factors, in a manner resembling the catalytic activity of negatively charged phospholipid surfaces [543a]. At the lipid bilayer surface, binding of factors IX, X, and prothrombin is dependent on Ca^{2+} , which forms a complex between negative charges on the phospholipid molecules and γ -carboxyglutamic acid residues present in these coagulation factors. The non-enzymatic protein cofactors V_a and $VIII_a$ are thought to interact with lipids by penetration into the hydrophobic membrane interior. This interaction does not depend on Ca^{2+} but requires negatively charged phospholipids. Various aspects of platelet procoagulant activity have been reviewed elsewhere [48, 53, 54, 543a, 728, 729, 731, 732, 733, 735, 737]

Prothrombin and factor X activation by platelets

The stimulatory effect of various platelet preparations in prothrombin and factor X activation (procoagulant activity) has been compared, using assay systems with highly purified coagulation factors and specific chromogenic substrates to measure rates of thrombin and factor X formation (Table I) [49, 50, 530, 543a]. ADP, PAF, serotonin, epinephrine and arachidonic acid have virtually no effect on procoagulant activity, and thrombin shows not more than a twofold increase in procoagulant activity relative to control platelets. Collagen is the only single physiological agonist capable of inducing a substantial increase in the capacity of platelets to stimulate prothrombin and factor X activation. Highest physiological stimulation, however, is achieved by the combined action of collagen and thrombin. The most potent stimulator of platelet procoagulant activity is the Ca^{2+} ionophore A23187. Treatment with diamide, an SH oxidizing compound, also renders platelets procoagulant. In contrast to other activators, this occurs at diamide concentrations where release and aggregation are no longer observed. The highest increase in pro-

coagulant activity is observed when platelets are completely lysed. The prothrombinase activity of lysed platelets equals that of their lipid extracts suggesting that no components other than phospholipids are responsible for this effect. The notion that phospholipids at the outer surface of activated platelets are responsible for the observed procoagulant activities is further supported by the observation that treatment of platelets with phospholipase A₂ from *N. naja* abolishes their procoagulant activity [51].

Expression of functional binding sites for coagulation factors on the platelet surface requires extracellular Ca²⁺ during platelet activation since no procoagulant activity is produced in the absence of Ca²⁺ or in the presence of Ca²⁺-channel blockers diltiazem and verapamil [52]. Treatment of platelets with prostacyclin or dibutyl-*c*-AMP, which stimulate cAMP-dependent processes, inhibits stimulation of procoagulant activity by collagen plus thrombin [734]. Aspirin, however, does not inhibit expression of procoagulant activity, indicating that thromboxane A₂ formation is not essential [734].

Stimulation of platelet procoagulant activity by the combined action of collagen and thrombin is dependent on certain properties of both agonists. Both collagen type I and type III are effective, and their activity can be abolished by a collagen-derived octapeptide, suggesting the requirement of specific ligand-receptor interactions [52]. The contribution of thrombin to the stimulation of procoagulant activity by collagen plus thrombin is dependent on its hydrolytic capacity. DFP-inhibited thrombin displays normal binding to platelets but lost its proteolytic activity, and is not effective in combination with collagen [730]. In the combination collagen plus thrombin, thrombin can be replaced by trypsin or α -clostripain without significant loss of procoagulant activity [730], supporting the notion that proteolysis of specific membrane proteins determines the synergistic activity of thrombin.

Changes in phospholipid exposure upon platelet activation

Determination of the orientation of phospholipids in plasma membranes is based on the

application of non-permeable lipid-reactive probes, which allow identification of the amount and possibly the lipid composition in the outer monolayer of the plasma membrane [192, 338, 476]. Exogenous phospholipases are such probes, which have the advantage over chemical reagents that a proper combination of them can react with all phospholipid classes and that reaction goes to completion [539, 540].

Determination of the maximal amount as well as the composition of phospholipids that can be degraded in the outer monolayer of the platelet plasma membrane has been achieved by a mixture of Sphingomyelinase and phospholipase A₂ [49, 50, 51]. With control platelets some 21% of the total phospholipids can be degraded in the absence of cell lysis, representing the phospholipid fraction in the external leaflet of the plasma membrane. This fraction is mainly composed of choline-phospholipids (SphM and PC), whereas degradation of amino-phospholipids (particularly PS) is limited (Table II). Activation of platelets by collagen or thrombin followed by addition of phospho-

Table I. Effect of different platelet treatments on their procoagulant activity, as expressed by activation of prothrombin and factor X.

platelet treatment	prothrombin activation	factor X activation
none	34	2
ADP	36	2
thrombin	40	3
collagen	98	18
collagen + thrombin	351	47
diamide	356	45
A23187	843	94
sonication	1170	149
collagen + thrombin followed by phospholipase A ₂	17	1

Prothrombin activation and factor X activation are expressed as nM thrombin per min and nM X_a per min, respectively. Data were obtained using purified coagulation factors under conditions where the procoagulant activity of platelets is rate limiting. Treatment specifications: 10 μ g/ml collagen, 2 nM thrombin, 5 mM diamide, 1 μ M A23187, 3 min direct-probe sonication. Data were taken from [50, 543a].

served when platelets are treated with A23187 before application of phospholipases. Remarkably, some 65% of all platelet phospholipids can be degraded in ionophore-treated platelets before the onset of cell lysis.

Changes in phospholipid composition of the outer membrane leaflet of platelets activated by thrombin are mainly caused by the release reaction, as a result of fusion of granular membranes with the plasma membrane. On the other hand, increased exposure of PS accompanied by decreased exposure of SphM in platelets activated by collagen plus thrombin, diamide, or A23187, presumably results from an enhanced transbilayer movement of phospholipids during platelet activation.

Since PS is the major anionic phospholipid in platelet membranes, its increased localization in the outer leaflet of the plasma membrane could be the cause of the increased procoagulant activity. When the percentage of total PS exposed at the outer surface of activated platelets is compared to the prothrombinase activity of the same activated platelets, the relation between exposure of PS and platelet procoagulant activity becomes evident (Fig. 7).

Prothrombinase activity of pathological platelets.

The ability of platelets to alter their membrane phospholipid orientation upon platelet activation, can be expected to require the participation of other platelet components or processes. Identification of these additional requirements may be derived from studies with different pathological platelets [48], since most of these are based on well-defined molecular or structural defects. Platelets which lack either α -granules (Gray platelet syndrome) or dense granules (Hermansky Pudlak syndrome) cannot be distinguished from normal platelets with respect to their activation-dependent procoagulant activity. This indicates that the release reaction may not be a prerequisite for the expression of platelet procoagulant activity. In view of the inability of thrombin to induce exposure of PS while complete release is achieved, it is suggested that the release reaction is not responsible for enhanced transbilayer movement of phospholipids in activated platelets.

Besides release, also platelet aggregation does not seem to be required for inducing a procoagulant surface. Platelets obtained from patients with Glanzmann's thrombasthenia fail

Table II. Stimulation-dependent changes in phospholipid composition of the outer monolayer of the platelet plasma membrane as determined by phospholipase treatment.

phospholipid class	unstimulated	thrombin	collagen	collagen + thrombin	diamide	A23187
PS	2 (4)	5 (15)	6 (19)	11 (46)	13 (49)	13 (75)
PE	10 (7)	16 (19)	17 (22)	36 (60)	26 (38)	36 (87)
PC	31 (17)	39 (34)	41 (37)	34 (39)	39 (41)	30 (50)
SphM	57 (63)	40 (69)	36 (65)	19 (43)	22 (45)	22 (75)
hydrolyzed fraction	21	33	35	45	40	65

Values are expressed as percentage of the fraction of total phospholipids which is hydrolyzed by the combined action of exogenous phospholipase A₂ and sphingomyelinase. This fraction represents the outer monolayer of the plasma membrane under non-lytic conditions. Relative standard deviations range from 20% for unstimulated platelets and platelets stimulated by thrombin (2 nM) or collagen (10 μ g/ml), to 10% for platelets treated with thrombin and collagen, diamide (5 mM), and A23187 (1 μ M). Values in parenthesis refer to the percent degradation of the whole phospholipid class. The contribution of PI (3% of total phospholipids) in addition to PS (12% of total phospholipids) is neglected. Abbreviations are explained in the text. Data are taken from [50].

be distinguished from normal platelets with respect to their activation-dependent procoagulant activity. This indicates that the release reaction may not be a prerequisite for the expression of platelet procoagulant activity. In view of the inability of thrombin to induce exposure of PS while complete release is achieved, it is suggested that the release reaction is not responsible for enhanced transbilayer movement of phospholipids in activated platelets.

Besides release, also platelet aggregation does not seem to be required for inducing a procoagulant surface. Platelets obtained from patients with Glanzmann's thrombasthenia fail to aggregate upon addition of collagen plus thrombin, but exhibit a similar increase in prothrombinase activity as observed with normal platelets. Since these platelets show partial or complete deficiencies in membrane glycoproteins II_b and III_a, the involvement of these proteins in mediating transbilayer movement of phospholipids over the platelet plasma membrane can be excluded.

Platelets from patients with Bernard-Soulier syndrome exhibit a particular interesting behavior regarding their procoagulant activity. In the unstimulated form, they are significantly more active than normal platelets, which cannot be explained by their increased size only. Bernard-Soulier platelets display abnormal phospholipid transbilayer asymmetry: the relative content of PS in the outer leaflet of the plasma membrane is higher than in normal platelets. This would explain the abnormally high basal level of procoagulant activity. Upon stimulation by collagen plus thrombin, procoagulant activity of Bernard-Soulier platelets increases towards similar levels as in normal platelets. Bernard-Soulier platelets are deficient in glycoprotein II_b and V. In addition the abnormally large size of these platelets may point to defects in cytoskeletal organization. Thus, cytoskeleton and glycoprotein II_b may be involved in maintaining low levels of procoagulant activity in unstimulated platelets.

Platelets lacking the glycoprotein I_a fail to respond to collagen with aggregation and release [455]. In addition, stimulation of these platelets by the combined action of collagen and thrombin did not increase procoagulant activity beyond basal levels, while the Ca²⁺ ionophore produced high levels of procoagulant activity as normal (unpublished results). It is con-

cluded that in these glycoprotein I_a deficient platelets the potency to develop a procoagulant surface is not diminished, but that stimulation of platelet procoagulant activity by collagen is inhibited. Thus, it is suggested that platelet glycoprotein I_a is important for collagen to induce procoagulant activity.

Most interestingly are results obtained from a patient with a moderately severe bleeding disorder, described as an isolated deficiency of platelet procoagulant activity [685]. Stimulation of these platelets resulted in a much lower number of binding sites for the prothrombinase complex as well as for the factor X activating complex than with normal platelets [543]. Incubations with phospholipases suggest a reduced exposure of PS upon platelet activation. This would point to a defect in the mechanism by which anionic phospholipids become exposed at the outer surface during platelet activation.

Possible mechanism of PS exposure.

Three possibilities can be distinguished which could explain the increased exposure of PS at the outer surface of activated platelets. First, PS may already be present at the outer

Fig. 7. Relation between prothrombinase activity and exposure of phosphatidylserine in activated human platelets. Each point represents the results from a different stimulation procedure. Unstimulated platelets are indicated by contr.

surface, but not available to chemical probes and proteins like coagulation factors and phospholipases, e. g. by interaction with membrane proteins. However, proteolytic treatment of platelets does not increase the availability of PS for chemical probes. Moreover, close to half of the plasma membrane phospholipids can be hydrolysed by exogenous phospholipases. Secondly, PS may be brought to the membrane surface during membrane fusion accompanying the release reaction. Such a mechanism does, however, not explain the difference in procoagulant activity of platelets activated by thrombin or by collagen plus thrombin. Thirdly, the increased exposure of PS results from increased transbilayer movement of phospholipids (flipflop). This mechanism seems most likely, since it would also explain the decrease in SphM content of the outer leaflet of the plasma membrane. Rapid transbilayer movement of phospholipids may involve flippases and may be dependent on cytoskeleton-membrane interaction, as discussed in a previous section.

Treatment of platelets and erythrocytes with phospholipase C from *C. welchii* results in formation of diacylglycerol which becomes phosphorylated to PA at the cytosolic side of

the plasma membrane. During treatment with phospholipase C substantial amounts of PS appear at the platelet surface and platelets become procoagulant [125]. The amounts of PA formed are of the same order as formed by the action of endogenous phospholipase C in activated platelets, suggesting that the amounts of diacylglycerol may produce non-bilayer structures by which enhanced flipflop can occur. It is emphasized that additional factors must play a role to explain why collagen and not thrombin enhances platelet procoagulant activity.

Involvement of the cytoskeletal proteins in the expression of platelet procoagulant activity is suggested by the results obtained with diamide. Treatment of platelets with diamide leads to extensive crosslinking of cytoskeletal proteins, particularly filamin, talin, myosin and actin [611]. In addition, PS becomes exposed and procoagulant activity increases [50]. More recently, examination of protein patterns obtained after gel electrophoresis showed that the prothrombinase activity of platelets activated by various agonists, correlated with the extent of proteolysis of cytoskeletal proteins by calpain (Fig. 8) [124]. In addition, platelets from a patient with a deficient generation of procoagulant activity displayed a diminished extent of degradation of cytoskeletal proteins [124]. These findings implicate cytoskeletal degradation by calpain to play a role in the expression of platelet procoagulant activity.

A model can be formulated which embodies both formation of non-bilayer structures and cytoskeletal degradation by calpain (Fig. 9). Formation of non-bilayer structures by diacylglycerol, as means of rapid transbilayer movement, is not sufficient to results in transport of phospholipids over the plasma membrane, regarding the inability of thrombin to render platelets procoagulant. Stimulation of calpain may be an additional prerequisite for flipflop of PS. It is conceivable that degradation of cytoskeletal proteins will cause alterations of cytoskeletal organization which may lead to a detachment of cytoskeleton from the plasma membrane. Assuming that platelet cytoskeletal proteins bind anionic phospholipids in the cytosolic leaflet of the plasma membrane, as is observed in erythrocytes, the detachment of the cytoskeleton from the plasma membrane would free the anionic lipids to participate in transbilayer movement along non-bilayer structures.

Fig. 8. Degradation of cytoskeletal proteins during stimulation of platelets by different procedures. Human platelets ($2 \cdot 10^7$ per ml) were stimulated by 2 nM thrombin, 10 μ g/ml collagen, 1 μ M A23187, or 10 μ M ADP. The arrowheads at the left designate the four major degradation products (see also Fig. 6). Indicated at the right are the positions of molecular weight standards in kDa. Picture taken from [124].

GLOSSARY OF THESIS CONTENTS

At the start of the research period during which the experiments presented in this thesis were performed, the prevailing model for the loss of transbilayer asymmetry of PS during the expression of platelet procoagulant activity included both disruption of cytoskeleton-membrane interactions and formation of non-bilayer structures, as outlined above.

Evidence was already obtained showing that the extent of proteolysis of cytoskeletal proteins by calpain correlated with the extent of transbilayer movement of PS. Based on these observations, the correlation between calpain activity and platelet procoagulant activity was further investigated by comparing their Ca^{2+} -dependence and their time-courses of generation after various platelet treatments. The results from these investigations are presented in chapter 2. In chapter 3, the relation between calpain activity and phospholipid transbilayer movement in platelets is further demonstrated,

making use of special properties of fluoride-treated platelets. Fluoride-treated platelets were also used to show that inhibition of platelet calpain leads to reduction of both cytoskeletal degradation and procoagulant activity. Additional evidence for the involvement of a decreased interaction between platelet cytoskeleton and plasma membrane in increased transbilayer movement of phospholipids came from experiments with artificially induced plasma membrane vesicles, as is shown in chapter 4.

Transbilayer movement of phospholipids in the platelet plasma membrane was investigated at the level of the lipid bilayer, employing the aspecific fluorescent membrane probe trimethylammonium-diphenylhexatriene (TMA-DPH). It was found that, besides a phospholipid transbilayer asymmetry, the platelet plasma membrane displays also a transmembrane fluidity gradient, as is described in chapter 5.

Fig. 9. Hypothetical model for the induction of phospholipid transbilayer movement in activated platelets. Phosphatidylserine (filled headgroups) is supposed to interact (arrows) with cytoskeletal proteins. Production of diacylglycerol (filled cones) will lead to formation of non-bilayer structures (inverted micelle), along which rapid flipflop may occur. A prerequisite for PS to participate in rapid flipflop is breakage of its interaction with cytoskeletal proteins.

Aspecific transbilayer movement of TMA-DPH enabled investigation of the occurrence of flip-sites in the platelet plasma membrane during platelet activation. These investigations showed that activation-dependent flipflop of PS in the platelet plasma membrane is accompanied by the formation of flipsites, as is shown in chapter 6.

Finally, in the general discussion, the route of intracellular mechanisms leading to platelet procoagulant activity is outlined, and molecular mechanisms resulting in formation of flipsites and the role of the platelet membrane skeleton therein will be discussed.

CHAPTER 2

Platelet procoagulant activity and cytoskeletal degradation by calpain.

ABSTRACT

The relationship between platelet calpain activity and platelet procoagulant activity was investigated by comparison of the time course of their generation after platelet stimulation by calcium ionophore A23187, or by the combined action of collagen and thrombin, or during exposure of platelets to the local anesthetics dibucaine or tetracaine. In addition, the Ca^{2+} dose-response curves of both activities in intact platelets, obtained by stimulation with A23187 in the presence of HEDTA/ Ca^{2+} buffers, were compared. Platelet procoagulant activity was determined by assaying for prothrombinase activity in the presence of saturating concentrations of factors X_a , V_a , and prothrombin. Platelet calpain activity was monitored by the degradation of its major substrates (filamin, talin, myosin) and the formation of their fragments as judged from protein patterns after gel electrophoresis. Platelet stimulation by A23187 resulted in a fast increase in prothrombinase activity, reaching its maximum level after about 20 seconds. Filamin and talin were completely hydrolysed within 15 seconds, and myosin was partly degraded between 15 and 30 seconds after platelet activation. When platelets were activated by collagen plus thrombin, prothrombinase activity was generated with a sigmoid time course, the steepest increase being observed between 1 and 2 minutes after platelet activation. Proteolysis of filamin and talin occurred between 0.5 and 1.5 minutes after platelet activation, while degradation of myosin became visible after 2 to 2.5 minutes. Dibucaine and tetracaine were both found to be potent stimulators of prothrombinase activity, with half maximal activities obtained at 0.7 and 2.8 mM resp. Using suboptimal concentrations of both local anesthetics, it was found that the generation of prothrombinase activity closely paralleled that of calpain activity over a time course of 1 hour. Ca^{2+} titration of intact platelets using A23187 and Ca^{2+} /HEDTA buffers, revealed half maximal response at about 15 μM free Ca^{2+} for both calpain and prothrombinase activity. These findings strongly suggest a causal relationship between generation of a procoagulant platelet surface and calpain-mediated degradation of filamin, talin, and myosin. Since an increased procoagulant activity reflects an increased exposure of phosphatidylserine at the platelet outer surface, the present findings suggest that platelet cytoskeletal proteins are involved in the regulation of membrane lipid asymmetry.

INTRODUCTION

One of the responses that platelets display upon activation is the generation of a procoagulant surface at the outside of the plasma membrane [54, 543, 730, 735]. This procoagulant surface greatly enhances the rate of two important sequential reactions in the coagulation cascade: the activation of factor X by a complex of factor IXa and factor VIIIa, and the activation of prothrombin by a complex of factor Xa and factor Va (the prothrombinase complex) [543]. The major determinant of platelet procoagulant activity is the membrane phospholipid phosphatidylserine, which upon activation becomes exposed at the outer surface of the platelet plasma membrane as a result of a transbilayer movement of phospholipids [49, 50, 543].

The agonist pattern of platelet procoagulant activity [50] is different from any other platelet response: collagen is the only single physiological agonist able to significantly increase platelet procoagulant activity. Higher stimulation of platelet procoagulant activity is achieved by the combined action of collagen and thrombin. The most potent stimulator of platelet procoagulant activity, however, is the Ca^{2+} ionophore A23187, indicative for the importance of increased cytosolic Ca^{2+} levels in this response. Investigation of the protein pattern from platelets activated by different agonists showed a strong correlation between the extent of expression of platelet procoagulant activity and the extent of proteolysis of cytoskeletal proteins by calpain[†] [124].

In view of findings that interactions between membrane skeletal proteins and anionic phospholipids may stabilize membrane lipid asymmetry in erythrocytes [168, 217, 256, 423, 572], it has been suggested that calpain-mediated degradation of cytoskeletal proteins may be a prerequisite for the loss of membrane lipid

asymmetry which occurs during the expression of platelet procoagulant activity [124].

In order to substantiate this suggestion, the correlation between stimulation of calpain activity and stimulation of platelet procoagulant activity was further explored by investigation of their time-response relationship after platelet activation by collagen plus thrombin, by A23187, and by exposure of platelets to the local anesthetics dibucaine or tetracaine, which have been reported to activate platelet calpain [119, 442, 608]. Furthermore, the Ca^{2+} response relationship of both activities was investigated, using Ca^{2+} buffers and A23187 to equilibrate extra- and intra-cellular free Ca^{2+} .

MATERIALS AND METHODS

Blood coagulation factors Va, Xa, prothrombin and thrombin were isolated and purified according to Rosing et al. [544]. Collagen type I from equine tendon was obtained from Hormon Chemie (München, Germany). The thrombin-specific chromogenic substrate, S2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine 6p-nitroanilide dihydrochloride), was purchased from Kabi Diagnostica (Stockholm, Sweden). Dibucaine-HCl and tetracaine-HCl were from ICN Pharmaceuticals (New York, USA) and from Sigma (St. Louis, USA) resp. Ionophore A23187 was obtained from Calbiochem-Hoechst (San Diego, USA). HEDTA (N-hydroxyethylethylenediaminetriacetic acid) was purchased from Sigma (St. Louis, USA). Other (bio)chemicals were of the highest grade commercially available.

Platelet isolation

Human platelets were isolated from freshly drawn blood as described previously [51]. Washed platelets were resuspended in a calcium-free buffer at pH 7.4, containing 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2 , 5 mM glucose and 0.1% fatty acid free human serum albumin (Hepes buffer). Platelet concentration was adjusted to approximately 10^8 ml^{-1} , using a Coulter Counter. Platelet lysis was determined by measuring lactate dehydrogenase activity in a 12000g supernatant according to Wroblewski and La Due [710].

Assay of platelet procoagulant activity

Platelet procoagulant activity was determined essentially as described by Rosing et al. [543], by assaying for prothrombinase activity under conditions at which the procoagulant surface is rate limiting. The assay was carried out at 37°C in Hepes buffer under continuous stirring (300 rev/min). To monitor the generation of prothrombinase activity after stimulation by collagen plus thrombin or A23187, factor Xa, factor Va, and CaCl_2 were added to prewarmed Hepes buffer 45 seconds before the start of

[†] The name calpain (E.C.3.4.22.17) is used for the ubiquitous Ca^{2+} -dependent neutral thiol-protease [431], originally described in platelets by Phillips and Jakabova [499]. The platelet protein known as P235 (M_r 235000) is named talin, in accordance with the demonstrated similarity between P235 and talin [470, 31]. The name filamin [683] is used for ABP (platelet actin-binding-protein, M_r 250000), considering their identical properties [212] and immunochemical cross-reactivity [472].

the assay. 15 Seconds later A23187 or collagen plus thrombin were added. Prothrombin was added 15 seconds before the assay was started by addition of platelets. Final concentrations: factor Xa 3 nM, factor Va 6 nM, prothrombin 4 mM, CaCl_2 3 mM, A23187 $1 \mu\text{M}$, collagen $10 \mu\text{g/ml}$, thrombin 4 nM, platelets $5 \times 10^6 \text{ ml}^{-1}$. After various time intervals, the activation of prothrombin was arrested by diluting a $25 \mu\text{l}$ aliquot of the assay mixture into a cuvette containing 1 ml buffer composed of 50 mM Tris/HCl, 100 mM NaCl, 2 mM EDTA, pH 7.5. Thrombin activity was determined spectrophotometrically at 405 nm, after addition of chromogenic substrate S2238 to a final concentration of $150 \mu\text{M}$. The amount of thrombin formed was calculated from the change in absorbance per minute, using a calibration curve made with active-site titrated thrombin.

To monitor the time-dependent effect of dibucaine and tetracaine on platelet procoagulant activity, platelets were incubated with local anesthetics at a count of $1 \times 10^8 \text{ ml}^{-1}$. After various time intervals a subsample was assayed for prothrombinase activity after dilution to $5 \times 10^6 \text{ platelets ml}^{-1}$. Thrombin formation was measured one minute after the addition of the subsample to the assay mixture containing factor Xa, factor Va, prothrombin, and CaCl_2 in Hepes buffer, as described for collagen plus thrombin and A23187.

Ca^{2+} titration of prothrombinase activity

All Ca^{2+} titrations were carried out at 37°C in a magnesium-free HEDTA/ Ca^{2+} buffer (10 mM Hepes, 120 mM NaCl, 3 mM KCl, 5 mM glucose, 30 mM HEDTA, various amounts of CaCl_2 , pH 7.4), at a platelet count of $2 \times 10^8 \text{ ml}^{-1}$ and under continuous stirring. An apparent K_d for HEDTA/ Ca^{2+} of $1.9 \mu\text{M}$ was determined under the same conditions as used for the Ca^{2+} titrations by computer fitting of the titration curve, obtained when a Ca^{2+} solution of known titer was titrated with a HEDTA solution, using a Ca^{2+} -selective electrode and a recording automatic titrator (Radiometer, Copenhagen, Sweden; type F2110Ca and RTS-system resp.). The free Ca^{2+} concentration of the various Ca^{2+} buffers was calculated using the apparent K_d for HEDTA/ Ca^{2+} , and was verified with the Ca^{2+} -selective electrode. It was found that the Ca^{2+} buffering capacity of the employed HEDTA/ Ca^{2+} buffer was sufficient to maintain applied extracellular free Ca^{2+} concentrations when platelets were challenged with A23187, over the whole range of extracellular free Ca^{2+} .

The Ca^{2+} dose-response relationship of platelet procoagulant activity was determined as follows: one minute after addition of 1 vol. platelets (suspended in 50 mM Hepes buffer, pH 7.4 at $2 \times 10^9 \text{ ml}^{-1}$) to 9 vol. HEDTA/ Ca^{2+} buffer, A23187 was added from a stock solution in DMSO to a final concentration of $0.3 \mu\text{M}$ (final DMSO concentration: 0.5%). Platelets were left to equilibrate with extracellular free Ca^{2+} for 5 minutes at 37°C under continuous stirring. Then formaldehyde was added to a final concentration of 300 mM. After 5 minutes a sample was taken and diluted into Hepes buffer containing 1% fatty acid-free human serum albumin and 3 mM Ca^{2+} to a platelet count of $5 \times 10^6 \text{ ml}^{-1}$. Prothrombinase activity was assayed by determination of the amount of

thrombin formed in one minute, after the addition of factor Xa, factor Va, and prothrombin.

The fixation of platelets after equilibration with the Ca^{2+} buffer is necessary to prevent post-activation of procoagulant activity by A23187-mediated entry of Ca^{2+} , which has to be present at millimolar concentrations during the prothrombinase assay for functional binding of the prothrombinase complex to a procoagulant surface. It was found that residual formaldehyde, present during the prothrombinase assay, inhibited prothrombinase activity by some 50%. This inhibition, however, was independent of the extent of prothrombinase activity itself, and independent of the free Ca^{2+} concentration of the Ca^{2+} buffer.

Determination of calpain activity by gel electrophoresis.

For proper correlation of calpain activity with prothrombinase activity, the same batch of platelets was used for both determinations throughout all experiments. Generation of calpain activity after platelet stimulation by A23187 or collagen plus thrombin was monitored at a higher platelet count than used for the prothrombinase assay, for practical reasons. The protein patterns were found not to be affected by the increased platelet count.

At 37°C , under continuous stirring, washed human platelets were added to human serum albumin-free Hepes buffer containing 3 mM CaCl_2 and the stimulators. After various time intervals calpain activity was arrested by addition of an equal volume of Hepes buffer, pH 6.6, containing 2 mM mersalyl acid, 2 mM phenylmethanesulphonyl fluoride, 2 mM benzamidine and 20 mM EGTA. After collection of platelets by centrifugation at $5000 \times g$ for 5 minutes in the presence of 15% acid-citrate-dextrose (180 mM glucose, 80 mM trisodium citrate, 52 mM citric acid), gel samples were prepared by dissolving the platelet pellet to a concentration of $2 \times 10^9 \text{ ml}^{-1}$ in sample buffer (62.5 mM Tris/HCl, 2% SDS, 10% glycerol, 3% mercaptoethanol, .005% bromophenolblue, 10 mM EGTA, 1 mM phenylmethanesulphonylchloride, 1 mM benzamidine, 1 mM mersalyl acid). Routinely all samples were boiled for 5 minutes before application to the gel. Disc electrophoresis was performed according to Laemmli [356], using 6-12% gradient polyacrylamide slab gels with a 3% polyacrylamide stacking gel.

Generation of calpain activity during incubation of platelets (10^8 ml^{-1} in Hepes buffer) with dibucaine or tetracaine, was followed by arresting and processing subsamples taken after various time intervals, as described for A23187 and collagen plus thrombin.

The effect of intracellular free Ca^{2+} concentration on platelet calpain activity was determined by equilibration of washed platelets with various HEDTA/ Ca^{2+} buffers in the presence of $0.3 \mu\text{M}$ A23187, as described for the determination of prothrombinase activity. After 10 minutes platelets were arrested and processed as described for collagen plus thrombin.

RESULTS

Generation of calpain- and prothrombinase-activity by A23187.

The Ca^{2+} ionophore A23187, in the presence of extracellular Ca^{2+} , is a very potent stimulator of platelet procoagulant activity: within one minute full activity is already expressed [530]. In order to monitor the generation of prothrombinase activity on such a short time scale, platelets were activated in the presence of all components of the prothrombinase complex, and samples were taken every 5 seconds to determine the amount of thrombin formed. In this way a time course of thrombin formation was obtained which initially increases exponentially, and then gradually develops into a straight line (Fig. 1A), reflecting the generation of prothrombinase activity to a constant level. Since this curve represents the time course of thrombin formation, the actual rate of thrombin formation (prothrombinase activity) at any time is reflected by the tangent of the curve at that time point. In this way the data were recalculated as prothrombinase activity relative to the final level (Fig. 1A). It was found that upon activation of platelets with A23187, prothrombinase activity is increased without any obvious delay and almost in a linear fashion, to reach its maximum level after about 20 seconds.

A complicating factor in the determination of prothrombinase activity on such short time scales, is the non-zero assembly time needed for the components of the prothrombinase complex to form a functional unit on the procoagulant surface. To overcome this problem, high concentrations of factor Xa and factor Va were applied (15 and 30 nM resp.). It was found that under these conditions the assembly time was negligible.

Platelet calpain activity was monitored by the degradation of its major substrates and the formation of their fragments as judged from protein patterns obtained after polyacrylamide gel electrophoresis of reduced protein samples. The major substrates of calpain are the cytoskeletal proteins filamin, talin, and myosin [124, 216, 499, 608, 656, 690]. The major degradation products are found at 190 kDa

(fragment 1, derived from filamin [212, 648] and talin [31, 212, 292, 470]), at 135 kDa (fragment 2, originating from myosin [124, 296, 492]), at 93 kDa (fragment 3, coming from filamin [212, 648]), and at 48 kDa (fragment 4, produced from talin [31, 212, 292, 470]). When platelets are activated by A23187 in the presence of 3 mM extracellular Ca^{2+} , calpain activity is already evident after about 10 seconds (Fig. 1B). Filamin and talin are most

Fig. 1. Generation of prothrombinase activity (A) and calpain activity (B) by A23187. (A): Generation of prothrombinase activity (●) was reconstructed from the time course of thrombin formation (▲) by determination of the slope of its tangent at different time points. Prothrombinase activity was assayed as described in the methods, with the exception that factor Xa and factor Va were present at resp. 15 and 30 nM. (B): Washed human platelets, at $1 \cdot 10^8 \text{ ml}^{-1}$, were stimulated by $1 \mu\text{M}$ A23187. Calpain activity is monitored after gel electrophoresis by the appearance of fragment 1 (from filamin and talin), fragment 2 (from myosin), fragment 3 (from filamin), and fragment 4 (from talin). The fragments are indicated at the right by numbered arrows. At the left the major cytoskeletal proteins are identified. The data shown are taken from a representative experiment.

rapidly degraded the next 5 seconds, followed by proteolysis of myosin.

When the generation of platelet procoagulant activity and calpain activity after stimulation by A23187 are compared, it is obvious that filamin and talin are completely degraded when half maximal prothrombinase activity is reached, while myosin degradation becomes apparent at maximal prothrombinase activity.

Generation of calpain- and prothrombinase-activity by the combined action of collagen plus thrombin.

Upon stimulation of washed platelets by both collagen and thrombin in the presence of Ca^{2+} and the factors of the prothrombinase complex, platelet procoagulant activity developed slowly during the first minute, followed by a steep increase between 1 and 2 minutes

after platelet stimulation, and gradually levelling off after 2 to 4 minutes (Fig. 2A). At this time scale the assembly time of the prothrombinase complex was not rate limiting.

Development of calpain activity was judged by the appearance of fragment 1 and fragment 2 (Fig. 2B). Most of fragment 1 was produced between 0.5 and 1.5 minutes after platelet stimulation, while fragment 2 appeared only faintly at about 2 minutes. Clearly, the degradation of filamin and talin by calpain coincides with the initial phase of generation of prothrombinase activity when platelets are stimulated by collagen plus thrombin.

Previously we have shown that when platelets are challenged with collagen plus thrombin in the presence of EDTA, proteolysis of cytoskeletal proteins by calpain does not occur [124]. Based on this observation we investigated the generation of both calpain- and procoagulant-activity after the addition of millimolar Ca^{2+} to platelets incubated with collagen plus thrombin for 5 minutes in the presence of 1 mM EGTA (Fig. 2A). It was found that immediately after the addition of Ca^{2+} , the prothrombinase activity steeply increased to its maximum level in 1.5 minute, while partial proteolysis of filamin and talin occurred during the first 20 seconds after the addition of Ca^{2+} (Fig. 2C).

Fig. 2. Generation of prothrombinase activity and calpain activity by the combined action of collagen plus thrombin. Development of prothrombinase activity and calpain activity was followed after platelet activation by collagen (10 $\mu\text{g}/\text{ml}$) plus thrombin (4nM), either in the presence of 3 mM Ca^{2+} (A, $\bullet$; B, resp.), or after addition of 4 mM Ca^{2+} to platelets previously activated with collagen plus thrombin for 5 minutes in the presence of 1 mM EGTA (A, $\blacksquare$; C, resp.). The lane identified by control (C) refers to untreated platelets. Incubations for gel electrophoresis were performed at a platelet count of $5 \times 10^7 \text{ ml}^{-1}$. Reference arrows aside the protein patterns are explained in Fig. 1. The data shown are taken from a representative experiment. Experimental details are outlined in the methods.

Generation of calpain- and prothrombinase-activity by local anesthetics.

To further explore the relationship between platelet calpain and procoagulant activity the local anesthetics dibucaine and tetracaine were employed. These agents seemed especially attractive, since they were reported to activate calpain [119, 442, 608], while they inhibit platelet aggregation and secretion [197]. First, their ability to generate platelet procoagulant activity was investigated at various concentrations. It was found that both dibucaine and tetracaine were potent stimulators of prothrombinase activity (Fig. 3). Dibucaine was 4 to 5 times more potent than tetracaine. Above 1 mM dibucaine or 3.5 mM tetracaine, measurements became unreliable as a result of considerable platelet lysis. Below these concentrations no significant lysis occurred.

To compare the generation of calpain- and procoagulant-activity by the local anesthetics, platelets were incubated with a suboptimal dose of dibucaine or tetracaine. In the presence of 0.5 mM dibucaine, platelet prothrombin-converting activity was raised to a maximum level within 45 minutes, with most of the increase after the first 10 minutes (Fig. 4A). When samples, taken from the same incubation, were investigated for calpain activity

Fig. 3. Dose-dependent stimulation of prothrombinase activity by the local anesthetics dibucaine (●) or tetracaine (■). Platelets ($5 \times 10^6 \text{ ml}^{-1}$) were challenged with the local anesthetics for 10 minutes before prothrombinase activity was determined one minute after addition of the components of the prothrombinase complex, as described in the methods. The data shown are obtained from a representative experiment.

Fig. 4. Generation of prothrombinase activity and calpain activity by dibucaine. The effect of dibucaine (0.5 mM) in the presence of 3 mM Ca^{2+} on the development of prothrombinase activity and calpain activity is shown in panel A and panel B resp. Panel C shows the effect of 1 mM EGTA on the stimulation of calpain by 1 mM dibucaine for 10 minutes (lane: + EGTA), in comparison with a similar stimulation in the presence of 1 mM Ca^{2+} (lane: + Ca^{2+}), and in comparison with untreated platelets (control lane). The data shown are taken from a representative experiment. A: Prothrombinase activity was assayed after dilution to 5×10^6 platelets ml^{-1} , as described in the methods. Untreated platelets retained a constant basal level of prothrombinase activity throughout the incubation period. B, C: reference arrows aside the protein patterns are explained in Fig. 1. Samples for gel electrophoresis were prepared as outlined in the methods.

(Fig. 4B), it was found that between 10 and 50 minutes after the addition of dibucaine, cytoskeletal degradation develops to its maximum level. This time course coincides with that of the generation of platelet procoagulant activity by dibucaine. When platelets were incubated with 2 mM tetracaine, a similar correlation as with dibucaine was observed between the generation of calpain activity and prothrombinase activity, on a time scale of one hour (not shown).

Incubation of washed platelets with 1 mM dibucaine for 10 minutes in the presence of 1 mM EGTA did not result in inhibition of prothrombinase activity (not shown), while cytoskeletal degradation by calpain was only moderately inhibited, in comparison to a similar incubation in the presence of 1 mM Ca^{2+} (Fig. 4C). When platelets were lysed by sonication in the presence of EGTA, subsequent addition of 1 mM dibucaine did not produce any degradation of cytoskeletal proteins (not shown). Therefore, activation of platelet calpain by local anesthetics is not dependent on extracellular Ca^{2+} , and is not a result of direct interaction with calpain.

Ca^{2+} dose-response relationship of the stimulation of calpain and procoagulant activity.

To gain additional support for a causal relationship between calpain- and procoagulant-activity, the response to the level of intracellular free Ca^{2+} of both activities was examined by stimulation of washed platelets with A23187 (0.3 μM), in the presence of HEDTA/ Ca^{2+} buffers at various free Ca^{2+} levels.

The dependence on free Ca^{2+} of the generation of platelet procoagulant activity, is shown in Fig. 5A. It was found that prothrom-

Fig. 5. Ca^{2+} dose-response relationship of platelet prothrombinase activity (A) and calpain activity (B). Washed human platelets, at a concentration of $2 \cdot 10^8 \text{ ml}^{-1}$, were challenged with 0.3 μM A23187 in the presence of 30 mM HEDTA/ Ca^{2+} buffer. Prothrombinase activity was determined after fixation with formaldehyde and dilution to $5 \cdot 10^6 \text{ platelets.ml}^{-1}$. Protein samples were prepared after arrest of calpain activity by addition of surplus EGTA and protease inhibitors. The data shown in (A) are the mean of the 3 experiments. The data shown in (B) are taken from a representative experiment. Experimental details are described in the methods.

binase activity increased slowly upon raising free Ca^{2+} from submicromolar levels. Between 5 and 30 μM free Ca^{2+} , prothrombinase activity developed with a steep increase. Half-maximal activity was obtained at 15 μM free Ca^{2+} . Beyond 30 μM free Ca^{2+} , prothrombinase activity gradually leveled off to reach maximal activity at 100 μM free Ca^{2+} .

Investigation of calpain activity over the same range of free extracellular Ca^{2+} levels (Fig. 5B), led to the following observations: (i) Hydrolysis of filamin and talin already starts at submicromolar levels of free Ca^{2+} as evidenced by the appearance of fragment(s) 1 and fragment 3. Major degradation of cytoskeletal proteins takes place going from 10 to 20 μM free Ca^{2+} , and between 20 to 25 μM free Ca^{2+} all filamin and talin are degraded. (ii) Hydrolysis of heavy-chain myosin rapidly increases just above 10 μM free Ca^{2+} , as judged from the appearance of fragment 2. Between 20 to 30 μM free Ca^{2+} myosin degradation is completed. (iii) In addition to the changes in the protein pattern with regard to the major cytoskeletal proteins filamin, talin, and myosin, several other proteins are affected by Ca^{2+} -dependent proteolysis. Noteworthy is the decrease in intensity of a protein band corresponding to the position of α -actinin (105 kDa), especially when free Ca^{2+} is raised above a level of 20 μM . Other Ca^{2+} -dependent changes in the protein pattern involving unidentified proteins are decreased bands corresponding to 170, 160, 130, 83, 75, 69, and 56 kDa, and new protein fragments at 140, 135, 125, 120, and 90 kDa (Fig. 5B). At all free Ca^{2+} levels the extent of protein degradation obtained after 15 minutes exposure to A23187 did not differ from those obtained after 5 minutes treatment with ionophore. Platelet lysis was insignificant at all Ca^{2+} concentrations. Generalizing, the most dramatic increase in Ca^{2+} -dependent proteolysis of platelet proteins is observed between 15 and 25 μM free Ca^{2+} . This level is quite similar to that at which half maximal activity of prothrombinase is observed.

DISCUSSION

The experiments presented in this study demonstrate that platelet calpain- and procoagulant-activity are closely related phenomena with regard to the time course of their development after stimulation of platelets with A23187 or collagen plus thrombin, or after exposure of platelets to the local anesthetics dibucaine or tetracaine. This parallel generation covers a time scale ranging from less than half a minute to one hour. The similarity between the Ca^{2+} -dependence of prothrombinase activity and calpain activity further strengthens their relationship.

The local anesthetics dibucaine and tetracaine appeared to be convenient agents to investigate the relationship between platelet procoagulant activity and calpain activity, since they were already known to stimulate calpain [119, 442, 608]. We found that local anesthetics are also able to stimulate platelet prothrombinase activity, supporting a finding by Peerschke [490] who used a clotting assay. The simultaneous stimulation of platelet prothrombinase activity and calpain activity by the local anesthetics strongly supports the notion that these two platelet processes are causally related.

It is generally observed that local anesthetics are inhibitors of other platelet activation phenomena [10, 197, 240, 377, 725]. More specifically, it has been shown that they inhibit phospholipase A₂ [196, 660] and protein kinase C [274, 659]. Therefore, the use of local anesthetics implicates that many processes normally involved in platelet activation (protein phosphorylation, arachidonate metabolism, secretion, aggregation) are not obligatory involved in the expression of platelet procoagulant activity. In fact, these implications have already been deduced from earlier experiments [48, 734].

The potent effect of Ca^{2+} ionophore A23187 both on calpain- and on prothrombinase-activity, suggests that uptake of extracellular Ca^{2+} may be required for both activities to develop. The role of extracellular Ca^{2+} is also obvious from the difference between the time course of development of prothrombinase activity of platelets triggered by collagen plus thrombin in the presence of Ca^{2+} , and of platelets to which Ca^{2+} was added after previous

activation by collagen plus thrombin in the presence of EGTA. In the second situation prothrombinase activity steeply increased immediately after the addition of Ca^{2+} , while in the first situation such a steep increase is only observed after a lag phase of about 1 minute. From these observations it is concluded that the entry of extracellular Ca^{2+} is an obligatory step in the generation of platelet procoagulant activity by collagen plus thrombin, and that the influx of this extracellular Ca^{2+} is enabled only after a delay of approximately 1 minute.

In contrast, the stimulatory effect of local anesthetics on calpain activity was independent of the presence of extracellular EGTA, and was not a result of direct activation of calpain. Therefore, Ca^{2+} must have been released from intracellular sources, though influx of extracellular Ca^{2+} , if present, may also occur [490]. Most likely, this intracellular source is membrane-bound Ca^{2+} , or more specifically lipid-bound Ca^{2+} being displaced by dibucaine or tetracaine [142, 315, 536].

Quantitative information about the levels of cytosolic free Ca^{2+} can be obtained from the Ca^{2+} titrations of platelet calpain- and procoagulant-activity, which both display the major increase at 10 to 15 μM free extracellular Ca^{2+} . The reliability of this information is based on the assumption that 0.3 μM A23187 will efficiently and accurately equilibrate cytosolic free Ca^{2+} with extracellular free Ca^{2+} . The validity of that assumption could not be assessed directly by measurements of the cytosolic level of free Ca^{2+} by Quin2 or Fura2, since the binding constants for Ca^{2+} of these fluorescent chelators do not allow accurate determination of free Ca^{2+} levels greater than 1 or 2 μM [250, 649, 650]. Jy and Haynes [304] used a low concentration of A23187 (0.5 μM) and a EGTA/ Ca^{2+} buffer system to manipulate cytosolic free Ca^{2+} . Using Quin2 they found that cytosolic free Ca^{2+} reflected extracellular free Ca^{2+} only in the range around the K_d of Quin2. In our experiments, accurate equilibration of cytosolic and extracellular free Ca^{2+} is suggested by the abundant transport capacity of A23187 as documented by the fast rise of calpain activity observed after addition of A23187 to platelets. In addition, halfmaximal activity of calpain is observed at about 15 μM free Ca^{2+} , as estimated from the protein patterns. This value is in excel-

lent agreement with values obtained with purified calpain-I.

Platelets are known to contain two isomers of calpain, calpain-I which is active at micromolar levels of free Ca^{2+} [212, 333, 552, 655, 656, 679], and calpain-II which is active at millimolar levels of free Ca^{2+} [648, 656, 720]. Halfmaximal activity of calpain-I is obtained at free Ca^{2+} levels ranging from 1 to 25 μM . Our experiments with intact platelets revealed Ca^{2+} -dependent cytoskeletal degradation with halfmaximal activity at about 15 μM free Ca^{2+} as estimated from the protein patterns, supporting the notion that Calpain-I is the major calcium-dependent protease in human platelets [656].

A peculiar finding, especially with regard to the Ca^{2+} titration of calpain activity in intact platelets is that degradation of cytoskeletal proteins stabilized within 5 minutes. It has been published that, upon stimulation, calpain is subject to autolysis of its heavy subunit [514], also in platelets [212], resulting in a reduction in molecular weight of the catalytic subunit. In addition it was observed that upon exposure to free Ca^{2+} , platelet calpain becomes very labile [552]. Therefore we assume that the termination of calpain activity in intact platelets within 5 minutes is due to autolysis, rendering calpain inactive. Another explanation could be that activated calpain becomes susceptible to inhibition by its endogenous inhibitor, calpastatin [134], also present in platelets [655].

The Ca^{2+} -dependence of prothrombinase activity would suggest that collagen plus thrombin raise cytosolic free Ca^{2+} to 10-15 μM . Activation of platelets by thrombin has been observed to result in cytosolic free Ca^{2+} levels ranging from 1 to 6 μM as reported by Quin2 [275, 525, 535, 554, 680] and Fura2 [509], or ranging from 7 to 12 μM as reported by aequorin [554, 680]. Activation of platelets by collagen has been shown to elevate cytosolic free Ca^{2+} levels to about 2 μM as monitored by Quin2 [680], or to levels ranging from 3 to 7 μM as reported by aequorin [448, 680]. Studies with electrically permeabilized platelets indicated that Ca^{2+} stimulated serotonin release is maximal at about 10 μM free Ca^{2+} [273, 327]. Taking these summarized data into account, a level of 10 to 15 μM cytosolic free Ca^{2+} , resulting from stimulation of platelets by collagen plus thrombin, seems feasible considering the

synergistic action of both potent platelet stimulators.

The close relationship between calpain activity and prothrombinase activity with regard to time course of development as well as Ca^{2+} requirement is consistent with a previous study by Comfurius et.al. [124]. They found the same order of potency for various platelet stimulators to evoke procoagulant activity and calpain-induced cytoskeletal breakdown. Extensive studies have demonstrated that prothrombinase activity reflects the presence of phosphatidylserine at the outer surface of the platelet plasma membrane [48, 50, 543, 733]. The present findings may therefore be interpreted as a relationship between exposure of phosphatidylserine and cytoskeletal degradation by calpain.

In erythrocytes a similar relationship between the loss of cytoskeletal integrity and the loss of asymmetric transbilayer distribution of phosphatidylserine is amply documented [104, 168, 217, 349, 423, 572, 703]. The major cytoskeletal protein of erythrocytes, spectrin, is thought to interact with anionic phospholipids [112, 404, 425], contributing to the maintenance of their asymmetric transbilayer distribution [256]. In addition, transbilayer reorientation of phosphatidylserine is thought to involve the formation of particular sites in the membrane, at which phosphatidylserine can take part in fast transbilayer movement, once released from binding to spectrin [39, 526]. A recent development with regard to the factors involved in regulation of transbilayer orientation of anionic phospholipids in erythrocytes, is the discovery

of an ATP-dependent inward transport of these lipids [144, 585, 643, 721].

In platelets, the regulation of transbilayer asymmetry of anionic phospholipids, in resting platelets as well as upon platelet activation, is poorly understood. As yet, interactions between the major platelet cytoskeletal proteins and anionic phospholipids have not been demonstrated directly. Bearer et.al. [28, 29] showed that submembrane cytoskeletal structures change their appearance with the same time course as the appearance of anionic lipid at the platelet outer surface, supporting the notion that in resting platelets interactions between structural proteins and anionic phospholipids play a role in the maintenance of transbilayer asymmetry of these lipids, analogous to erythrocytes. The experiments presented in this paper and in a previous one [124], show that degradation of cytoskeletal proteins by endogenous calpain could disturb these cytoskeleton-membrane interactions, contributing to a loss of membrane lipid asymmetry. This suggests a role for calpain in the regulation of the mechanisms by which phosphatidylserine becomes exposed at the outer platelet surface thus increasing the procoagulant activity of platelets.

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CHAPTER

3

Relation between calpain and phospholipid transbilayer movement in fluoride-treated platelets.

ABSTRACT

Treatment of platelets with fluoride (10 mM) was found to result in a transient increase in Ca^{2+} -permeability of the platelet plasma membrane. This phenomenon was used to provide supplementary evidence for the suggestions made earlier [Comfurius et.al.(1985), *Biochim.Biophys.Acta*, 903: 206; Verhallen et.al.(1987), *Biochim.Biophys.Acta*, 815: 143], that cytoskeletal disruption by calpain is involved in the process leading to transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity. This was achieved by relating both calpain activity and exposure of phosphatidylserine with platelet procoagulant activity. It was found that only upon addition of extracellular Ca^{2+} to fluoride-treated platelets, procoagulant activity, expressed as prothrombinase activity, and calpain activity, estimated from protein patterns after gel electrophoresis, were generated. Both Ca^{2+} -inducible prothrombinase activity and calpain activity followed an identical time course during incubation with fluoride: after a time lag of about 10 min they sharply increased towards a peak level. Upon further incubation with fluoride, both activities decreased towards a final plateau, still above basal level. The presence of leupeptin during incubation with fluoride was found to inhibit Ca^{2+} -inducible calpain activity and prothrombinase reactivity in an identical way. Ca^{2+} -inducible exposure of phosphatidylserine, as determined with extracellular phospholipase A_2 , showed a similar pattern as Ca^{2+} -inducible calpain activity and prothrombinase activity. From the strict parallelism between prothrombinase activity, calpain activity and exposure of phosphatidylserine, it is concluded that calpain plays an important role in the activation-dependent transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity. It is suggested that degradation of the platelet membrane skeleton by calpain disturbs the structural organization of the lipid bilayer of the platelet plasma membrane leading to enhanced transbilayer movement of phospholipids and appearance of phosphatidylserine at the platelet outer surface.

INTRODUCTION

Blood platelets have a multifaceted function in the hemostatic process. They initiate hemostatic plug formation upon vessel wall injury, and they promote fibrin formation by the coagulation cascade. In order to perform these functions platelets exhibit a wide variety of cellular responses. Primary hemostatic plug formation requires platelet adhesion, aggregation, and exocytosis. Activated platelets accelerate coagulation by increasing the rate of two sequential coagulation reactions: conversion of factor X to X_a by a complex of factors IX_a and $VIII_a$, and the conversion of prothrombin to thrombin by a complex of factors X_a and V_a (prothrombinase complex). This property of stimulated platelets, referred to as procoagulant activity, is catalytic in nature [543a, 544], and can be interpreted as an increased number of binding sites for both the factor X activating complex and the prothrombinase complex [543a, 737].

In previous papers, we presented extensive evidence that anionic phospholipids present at the outer surface of activated platelets are the major determinant of the binding sites for the prothrombinase complex [53, 732, 733]. Using phospholipid vesicles of various compositions, it was suggested that phosphatidylserine is the physiologically relevant negatively charged phospholipid in both coagulation reactions [53, 162, 529, 543a, 729]. This notion was further substantiated by the effect of phospholipases on the procoagulant activity of resting, and stimulated platelets. These studies showed that only those phospholipases that can hydrolyse phosphatidylserine will decrease platelet procoagulant activity [51, 543a], and that stimulation of platelet procoagulant activity by a variety of agonists is related to the amount of phosphatidylserine exposed at the outer surface after platelet activation [50].

The plasma membrane of a resting platelet displays an asymmetric distribution of phospholipids [50, 98, 494, 569, 729]. Most extreme is the distribution of phosphatidylserine and sphingomyelin, which are almost exclusively located at the cytoplasmic and extracellular face of the platelet plasma membrane, respectively. To explain the increased exposure of phosphatidylserine after stimulation of platelet procoagulant activity, it is assumed that trans-

bilayer movement of phospholipids (flipflop) is greatly enhanced during stimulation of platelet procoagulant activity [50, 730]. This flipflop process is thought to produce randomization of phospholipids in the bilayer membrane, since in the outer leaflet exposure of phosphatidylserine is counterbalanced by a loss of sphingomyelin.

At present, the cellular mechanisms responsible for increased exposure of phosphatidylserine during expression of platelet procoagulant activity are poorly understood. In search of such mechanisms, we observed a striking correlation between the potency of various agonists to induce exposure of phosphatidylserine and to stimulate platelet calpain (endogenous calcium-dependent protease, EC.3.4.22.17) resulting in degradation of major cytoskeletal proteins (filamin, talin, and myosin) [124]. The relation between platelet procoagulant activity and calpain activity was further documented by comparing the time course of their development, their dose-response relationship, and their Ca^{2+} dependence [664].

During studies concerning the long term effect of metabolic ATP depletion on transbilayer phospholipid asymmetry in platelets, using sodium fluoride as a metabolic inhibitor, we observed that during incubation with fluoride for several hours, platelets temporarily exhibited an increase in platelet procoagulant activity. This reversible time course appeared to reflect a transient increase in Ca^{2+} -permeability of the platelet plasma membrane during incubation with fluoride. In this paper, the reversibility of fluoride-dependent Ca^{2+} -inducible platelet procoagulant activity is used to provide supplementary evidence for the suggestions made earlier that calpain activity coincides with exposure of phosphatidylserine. This is achieved by relating both calpain activity and exposure of phosphatidylserine with platelet procoagulant activity expressed as prothrombinase activity. In addition, the effect of leupeptin (an oligopeptide inhibitor of calpain) on the relation between calpain- and prothrombinase-activity was explored.

MATERIALS AND METHODS

Sodium fluoride was from Merck (Darmstadt, FRG). Leupeptin, (acetyl-L-leucyl-L-leucyl-L-arginal) was

from Sigma (St. Louis, USA). Blood coagulation factors Va, Xa, prothrombin were isolated and purified from bovine blood according to established procedures described elsewhere [543a]. S2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride), was from Kabi Diagnostica (Stockholm, Sweden). Phospholipase A₂ (phosphatide-2-acylhydrolase: EC 3.1.1.4) was purified according to Zwaal et al. [736] from bee venom (*Apis mellifica*) obtained from Koch Light Laboratories (Colnbrook, UK). FURA-2 AM was from Molecular Probes Inc (Eugene OR, USA).

Platelet isolation

Washed human platelets were isolated by differential centrifugation as described previously [51] from fresh blood, drawn from healthy volunteers receiving no medication. Blood was anticoagulated with acid-citrate-dextrose. Washed platelets were resuspended in an isotonic buffer at pH 7.4, containing 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 5 mM glucose and 0.05% fatty acid-free human serum albumin (Hepes buffer). Platelet concentration was inferred from their absorption at 405 nm using a calibration curve determined with a Coulter Counter. Platelet lysis was determined by assaying lactate dehydrogenase (EC 1.1.1.27) in a 12000g supernatant according to Wroblewski and La Due [710].

Measurement of platelet procoagulant activity

Platelet procoagulant activity was determined as described earlier [543a, 664] by assaying prothrombinase activity under conditions at which the procoagulant surface is rate limiting. The assay was carried out at 37°C in Hepes buffer under gentle stirring (250 rev/min). To monitor the effect of fluoride on platelet procoagulant activity, platelets (2×10^8 ml⁻¹) were incubated with 10 mM fluoride at 37°C, without stirring to prevent aggregation. Immediately before the addition of fluoride, 1 mM EGTA was added to sequester trace amounts of Ca²⁺. At different time intervals a 15 µl aliquot of the fluoride incubation was diluted twentyfold into Hepes buffer containing 3 mM CaCl₂. No change in pH was apparent upon this dilution. After 1 min factor Va (6 nM final conc.) and factor Xa (3 nM final conc.) were added, and 1 min later the prothrombinase reaction was started by addition of prothrombin (final conc. 4 µM, final assay volume 0.5 ml). The amount of thrombin formed 1 min after the addition of prothrombin was enzymatically determined using the thrombin-specific chromogenic substrate S2238.

Occasionally, the time course of thrombin formation after Ca²⁺-addition was monitored by assaying thrombin activity in an aliquot taken every 10 sec after addition of fluoride-treated platelets to the complete prothrombinase mixture.

Determination of Ca²⁺ influx using Fura-2

Platelets were loaded with Fura-2 according to Rao et al. [525]. Platelets pelleted from platelet rich plasma were resuspended in Hepes buffer to approximately 3×10^8 ml⁻¹ and incubated with 1 µM Fura-2 AM for 30 min at 37°C. After loading, platelets were washed as described under platelet isolation. The effect of fluoride on Ca²⁺-

influx into Fura-2 loaded platelets was monitored at different time intervals after tenfold dilution in Hepes buffer of an aliquot of platelets incubated with fluoride (2×10^8 pl.ml⁻¹, 10 mM fluoride, 1 mM EGTA, 37°C). 3 mM Ca²⁺ was added under continuous high speed recording (5 sec per inch) of Fura-2 fluorescence. The initial rate of change in fluorescence signal after Ca²⁺ addition was taken to reflect quantitatively the rate of Ca²⁺ influx. Fluorescence intensities (F) were recorded at 37°C under continuous stirring (400 rev/min) with an SLM Aminco SPF-500C fluorimeter equipped with a light-tight injection port and a stirring device (Hellma). Excitation and emission wavelengths were set at 335 and 500 nm with 5 and 20 nm slits, respectively.

Determination of calpain activity by gel electrophoresis

Calpain activity in platelets was estimated from protein patterns after gel electrophoresis, by monitoring degradation of substrates and formation of fragments, as described in detail [664]. Samples for gel electrophoresis were taken simultaneously with samples for the prothrombinase assay, from the same incubation of platelets with fluoride, at various time intervals. They were diluted tenfold in albumin-free Hepes buffer, containing 3 mM Ca²⁺, after 3 min calpain activity was arrested by addition of Hepes buffer containing protease inhibitors and excess EGTA. Disc electrophoresis was performed according to Laemmli [356] on 6-12% gradient polyacrylamide slab gels. Gels were stained with Coomassie brilliant blue. Quantitative analysis of protein patterns was performed by densitometric scanning of stained gels (LKB 2202 Ultrascan laser densitometer, equipped with an integrator). Calpain activity was expressed as the amount of fragments (at 190, 135 and 95 kDa) relative to total protein. To eliminate differences due to differences in amount of total protein, the amount of actin was taken as an internal standard.

Determination of exposure of phosphatidylserine

The exposure of phosphatidylserine was inferred from its accessibility towards excess extracellular phospholipase A₂ from bee venom. Samples for phospholipase treatment were taken simultaneously with samples for the prothrombinase assay from the same incubation of platelets with fluoride (5×10^8 pl.ml⁻¹, 10 mM fluoride, 1 mM EGTA). After tenfold dilution into Hepes buffer containing 10 mM Ca²⁺, phospholipase A₂ was added to a final concentration of 2 IU.ml⁻¹. Every 4 min a subsample (10^9 platelets) from the phospholipase A₂ incubation was taken for examination of phospholipid degradation and determination of platelet lysis. Phospholipase A₂ activity was inhibited by addition of 20 mM EGTA to the subsample, prior to the extraction of lipids according to Bligh and Dyer [61]. Phospholipid analysis was carried out by two-dimensional thin layer chromatography followed by phosphorus determination as described by Verkleij et al. [666].

RESULTS

Fluoride-dependent Ca^{2+} -induced platelet procoagulant activity

Changes in Ca^{2+} -permeability of the platelet plasma membrane during incubation with fluoride were investigated employing Fura2-loaded platelets. The initial change in Fura2 signal after addition of extracellular Ca^{2+} was taken to represent Ca^{2+} -influx quantitatively. It was found that the entry of extracellular Ca^{2+} into the platelet cytosol was transiently facilitated during the course of a two-hour incubation of platelets with fluoride (Fig. 1A).

The effect of fluoride (10 mM) on platelet procoagulant activity was determined by assaying prothrombinase activity in subsamples taken from an incubation of platelets with fluoride. It was found (Fig. 1B) that during incubation with fluoride prothrombinase activity sharply increased, after a time lag of 10 to 15 min, to a maximum level of approximately 700 nM thrombin per min. After reaching this maximum, prothrombinase activity gradually decreased upon progressive incubation with fluoride, to level off at about one-third of its peak level. Except for the time lag, the transient character of Ca^{2+} -inducible prothrombinase activity coincides with the transient increase in Ca^{2+} -permeability during fluoride treatment. Under the same conditions of fluoride treatment, platelet aggregation and ^3H -serotonin release started after a delay of 5 minutes and levelled off at about 10% and 65%, respectively, within 15 to 20 min after addition of fluoride.

It is emphasized that fluoride treatment is carried out in the absence of extracellular Ca^{2+} , while prothrombinase activity is obligatory measured in the presence of extracellular Ca^{2+} . Considering the importance of extracellular Ca^{2+} for the expression of platelet procoagulant activity [124, 664] and the effect of fluoride treatment on Ca^{2+} permeability of the platelet plasma membrane, the question arose whether the observed procoagulant activities were induced during incubation of platelets with fluoride in the absence of Ca^{2+} , or whether they were induced by influx of extracellular Ca^{2+} present during the prothrombin-

ase assay. Therefore, the time course of thrombin formation during the prothrombinase assay was determined and recalculated as generation of prothrombinase activity [664]. It appeared that the procoagulant activity of fluoride-treated platelets was actually generated by extracellular Ca^{2+} during the prothrombinase assay, reaching its maximum level within 2 min (inset Fig. 1B), i.e. before addition of prothrombin.

Fig. 1. Time course of Ca^{2+} -influx (A) and prothrombinase activity (B) during incubation of platelets with fluoride. Both parameters were evaluated after addition of 3 mM Ca^{2+} to platelets incubated with 10 mM fluoride, as described under Methods. The curve for Ca^{2+} -influx (A), represented by the initial change in Fura-2 fluorescence ($\Delta F_{\text{fura}}/\text{min}$) after Ca^{2+} addition, is the mean ($\pm$ SEM) of 5 experiments. The curve for prothrombinase activity (B) was constructed from 9 experiments in duplo (mean $\pm$ SEM). In general, the reversible time course of both effects of fluoride on platelets was highly reproducible, though peak-time and peak-width differed between different platelet preparations. Platelet lysis was negligible during the two hours incubation with fluoride. *Inset (B):* Generation of prothrombinase activity immediately after addition of Ca^{2+} to platelets incubated with fluoride for 30 min. Ordinate: prothrombinase activity expressed as percentage of maximal activity. Abscissa: time (sec) after Ca^{2+} addition. It appeared that throughout the whole incubation of platelets with fluoride, Ca^{2+} -induced generation of prothrombinase activity followed the same time course (not shown).

Fluoride-dependent Ca^{2+} -induced exposure of phosphatidylserine.

Phospholipase A_2 from bee venom was used to determine the amount of phosphatidylserine exposed at the outer surface of fluoride-treated platelets after Ca^{2+} -exposure. This approach is based on the observation that under non-lytic conditions only phospholipids present in the outer leaflet of the plasma membrane can be hydrolysed by exogenous phospholipase A_2 [50, 98, 539, 736]. Fluoride-dependent Ca^{2+} -induced exposure of phosphatidylserine was related with prothrombinase activity at three characteristic time points during fluoride treatment: at the start, at the peak level and at the lower final level of prothrombinase activity. In order to determine the saturating level of hydrolysis by phospholipase A_2 , the time course of hydrolysis of both phosphatidylserine and total phospholipids was determined. In this way changes in hydrolysis of phosphatidylserine can be compared with changes in hydrolysis of total phospholipids. It was found that at peak levels of prothrombinase activity, accessibility of phosphatidylserine towards phospholipase A_2 was

Fig. 2. Hydrolysis of phosphatidylserine (A) and total phospholipids (B) in fluoride-treated platelets, after exposure to Ca^{2+} . Degradation of phospholipids in the outer membrane of fluoride-treated platelets by phospholipase A_2 from bee venom was examined 0 min (Δ), 30 min ($\bullet$) and 120 min ($\circ$) after the addition of fluoride, as outlined in the Methods. The data shown are the mean ($\pm$ SEM) of 3 experiments. During treatment with phospholipase A_2 , platelet lysis gradually increased to a maximum of 10%. Data shown were corrected for phospholipid hydrolysis in lysed cells [124]. The presence of residual fluoride during treatment with phospholipase A_2 was found not to affect phospholipase A_2 activity.

dramatically increased to approximately 65%, compared to less than 1% and 10% at the start and at the end of the incubation with fluoride, respectively (Fig. 2A). In contrast, hydrolysis of total phospholipids was only two-fold increased at peak level prothrombinase activity in comparison with the hydrolysis at the end of fluoride treatment (Fig. 2B). At the start of fluoride treatment hardly any hydrolysis of phospholipids was observed, in agreement with earlier observations [49, 98].

Fluoride-dependent Ca^{2+} -induced calpain activity

The relationship between calpain activity and prothrombinase activity was determined by applying a similar Ca^{2+} -exposure after fluoride treatment. From the protein patterns (Fig. 3C) and their quantitative analysis (Fig. 3B), it is obvious that calpain activity followed the same reversible time course as prothrombinase activity: a fast rise after a time lag, followed by a gradual decline. In addition, when extracellular Ca^{2+} was omitted, no calpain activity could be demonstrated in fluoride-treated platelets (Fig. 3C). Generation of prothrombinase activity in fluoride-treated platelets was similarly dependent on extracellular Ca^{2+} (Fig. 1B).

In search of a direct demonstration of the involvement of calpain in Ca^{2+} -activated platelet procoagulant activity, we investigated the effect of leupeptin (inhibitor of calpain) on the relation between fluoride-dependent Ca^{2+} -induced prothrombinase activity and calpain activity. It has been suggested that leupeptin crosses the platelet plasma membrane [21, 548]. The presence of leupeptin (0.5 mM) during incubation of platelets with fluoride resulted in a significantly decreased peak level of calpain activity, and the final low level was reached faster (Fig. 3B,C). An identical effect of leupeptin was observed on prothrombinase activity of platelets sampled from the same incubation with fluoride (Fig. 3A). Considering that leupeptin can inhibit thrombin [547a], the effect of leupeptin on the prothrombinase assay was investigated. At the concentration used (15 μM in the prothrombinase assay), leupeptin had no effect on the enzymatic activity of the prothrombinase complex in the presence of vesicles composed

of 20 mole% phosphatidylserine and 80 mole% phosphatidylcholine (data not shown).

The parallelism between Ca^{2+} -inducible prothrombinase activity and calpain activity was consistently observed ($n=8$). The effect of leupeptin on both prothrombinase activity and calpain activity was highly variable between different platelet preparations, and frequently difficult to demonstrate. However, on those occasions that leupeptin did exhibit a clear effect ($n=4$), the time course of both Ca^{2+} -induced prothrombinase activity and calpain activity during incubation of platelets with fluoride was identically affected.

DISCUSSION

In this paper we used fluoride-treated platelets to extend previous observations suggesting a role for calpain in the stimulus-response coupling of platelet procoagulant activity [124, 664]. During incubation with fluoride the

Ca^{2+} -permeability of the platelet plasma membrane was increased in a reversible manner, causing platelet procoagulant activity, calpain activity, and exposure of phosphatidylserine to be enhanced with a similar time course.

At present the action of fluoride is not fully understood. Uptake of fluoride by platelets at physiological pH is maximal within 10 minutes [436], indicating that the effect of fluoride on Ca^{2+} -permeability of the platelet plasma membrane during a two hour incubation should result from cellular processes secondary to fluoride. Fluoride is known to affect platelets in at least two different ways: 1) glycolysis is instantaneously and efficiently inhibited, leading to a fast decrease in metabolic ATP [435, 436, 437], and 2) it activates various G-proteins, resulting in platelet activation [76, 317, 504]. Considering the irreversibility of fluoride-induced ATP depletion, the activation of G-proteins seems to be the best candidate to explain the reversible effect of fluoride on Ca^{2+} -permeability of the platelet plasma membrane.

Fig. 3. Relation between Ca^{2+} -induced prothrombinase activity (A) and calpain activity (B,C) of fluoride-treated platelets, and the effect of leupeptin on this relation. Platelets were incubated with 10 mM fluoride in the absence (●) or presence (○) of 0.5 mM leupeptin. (B): Degradation of cytoskeletal proteins by calpain after addition of Ca^{2+} to fluoride-treated platelets, as judged by the formation of their fragments (sum of protein bands indicated at the right side of (C)). Data were quantitated from the gels shown in (C), after densitometric scanning as outlined in the methods. Changes in the amount of fragments were counterbalanced by changes in the amount of substrates (not shown). (C) Upper and lower panel: time course of Ca^{2+} -inducible calpain activity during incubation with 10 mM fluoride, in the absence and presence of 0.5 mM leupeptin, respectively. Calpain activity is indicated by the disappearance of its substrates (indicated at the left), and the appearance of their fragments (indicated at the right). Samples for gel electrophoresis were taken from the same incubation as that of which the Ca^{2+} -induced prothrombinase activity was determined. For experimental details is referred to the Methods. The results shown in this figure are taken from a representative experiment.

Fluoride has been reported to increase platelet cytosolic Ca^{2+} levels, partly dependent of extracellular Ca^{2+} [76, 324, 504]. Also in other cells fluoride has been shown to promote Ca^{2+} -influx [183, 355, 619]. In addition, G-proteins are suggested to be involved in the regulation of Ca^{2+} -channels [244, 282, 311, 355], and, in general, ion-channels form part of the effector targets of G-proteins in cellular signal transduction [294]. Therefore, we assume that the transient facilitation of Ca^{2+} -influx by fluoride is caused by the action of fluoride on G-protein dependent Ca^{2+} -channels in the platelet plasma membrane.

The involvement of G-proteins could explain the reversible nature of fluoride-dependent Ca^{2+} -permeability of the platelet plasma membrane. Activation of G-proteins by fluoride has been shown to result from the action of aluminiumtetrafluoride (AlF_4^-) [614], suggested to fill the space of the third phosphate group in the guanine-nucleotide binding pocket containing already GDP in the inactive state [57]. Extensive depletion of phosphorylated nucleotides during prolonged incubation with fluoride could result in depletion of GDP. Resynthesis of ATP from IMP at the cost of GTP has been demonstrated in fluoride-treated platelets [438]. Thus, it is possible that the decline in fluoride-dependent Ca^{2+} -permeability of the platelet plasma membrane, after reaching peak level, is due to depletion of GDP resulting in inactivation of fluoride-stimulated G-proteins and closure of Ca^{2+} -channels.

Ca^{2+} -permeability increases right from the start of fluoride treatment. However, calpain activity and prothrombinase activity were increased only after a time lag of 10 to 15 min. Platelet aggregation and release during fluoride treatment were found to level off within 15 to 20 min, in agreement with earlier reports [317, 436, 504]. This suggests that prothrombinase activity and calpain activity are dependent on higher cytosolic free Ca^{2+} levels than required for aggregation and release, confirming previous findings [664]. In addition, the discrepancy between the development of platelet aggregation and release, and the development of prothrombinase activity and calpain activity upon fluoride treatment, indicates that the cellular mechanisms generally shown to be involved in platelet stimulus-response coupling (phospholipase C, phospholipase A2 and pro-

tein kinase C) are not directly responsible for the generation of increased phospholipid transbilayer movement during the expression of platelet procoagulant activity. This, is suggested by the activation of phospholipid hydrolysis [76, 317, 504] and protein phosphorylation [317, 438] by fluoride during the first 10 to 20 min of incubation of platelets with fluoride, where Ca^{2+} -inducible calpain activity and prothrombinase activity are not increased. These conclusions are in accordance with previous observations that thrombin, being a strong platelet agonist, is hardly able to induce procoagulant activity and calpain activity [124].

Peak level of fluoride-dependent Ca^{2+} -induced prothrombinase activity is accompanied by increased accessibility of phosphatidylserine towards phospholipase A2 in comparison with unstimulated platelets and in comparison with platelets treated with fluoride for two hours. This increased accessibility of phosphatidylserine cannot be explained only by increased hydrolysis of total phospholipids. Thus, it is concluded that the ratio phosphatidylserine/total phospholipids in the outer monolayer of the platelet plasma membrane follows a similar reversible time course as the fluoride-dependent Ca^{2+} -induced prothrombinase activity. It is unlikely that exposure of phosphatidylserine results from fusion of granular membranes with the plasma membrane during the release reaction, since the release precedes the development of prothrombinase activity in fluoride-treated platelets. Therefore phosphatidylserine must have crossed the lipid bilayer of the platelet plasma membrane considering its location in unstimulated platelets [50, 98, 494, 569].

The observation that phospholipase A2 alone is able to hydrolyse phospholipids in the platelet plasma membrane only under those conditions where an increased prothrombinase activity is seen, confirms earlier findings [50]. The ability of phospholipase A2 to hydrolyse membrane lipids is greatly dependent on the molecular packing of the lipids, expressed as surface pressure, and may be enhanced by the presence of anionic phospholipids [157]. Increased ability of phospholipase A2 to hydrolyse membrane lipids has been shown to correlate with increased merocyanine-540 uptake of the membrane [95, 103, 526], indicating a decreased order of membrane lipids. Therefore, we suggest that the increased hydrolysis of total

phospholipids at peak level of fluoride-dependent Ca^{2+} -induced prothrombinase activity reflects both changes in membrane lipid organization, as they are thought to occur during enhanced transbilayer movement of phospholipids [154], and the presence of anionic phospholipids in the outer monolayer of the platelet plasma membrane.

The observation that fluoride-dependent Ca^{2+} -induced calpain activity follows the same reversible time course as prothrombinase activity, clearly extends previous observations suggesting a relation between calpain activity and platelet procoagulant activity [124, 664]. In particular, the identical effect of leupeptin on both fluoride-dependent calpain activity and prothrombinase activity provides a strong argument for a role of calpain in the transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity, considering that calpain is the major Ca^{2+} -dependent protease in platelets [648].

The relation between calpain activity and exposure of phosphatidylserine does not unequivocally imply that cytoskeletal proteins are the important substrates of calpain involved in regulation of transbilayer movement of phosphatidylserine. However, it has been shown (1) that the substrate specificity of calpain is mainly confined to cytoskeletal proteins, in general [431, 514, 682] as well as in platelets [216, 499, 608, 656, 690], and (2) that the organization of cytoskeletal proteins plays an important role in the regulation of transbilayer asymmetry of anionic phospholipids in the erythrocyte [217, 256, 423]. Therefore, we suggest that it is the degradation of cytoskeletal proteins by calpain which is causally related to transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity.

The involvement of calpain mediated cytoskeletal degradation in the regulation of transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity can be understood by applying the current model of the regulation of phospholipid transbilayer asymmetry in the erythrocyte. In erythrocytes the asymmetric distribution of phospholipids over the plasma membrane is thought to be maintained 1) by interaction of the lipid bilayer with cytoskeletal proteins [168, 256] located underneath the plasma membrane (membrane skeleton) [72, 114, 249, 402], and 2) by the ATP-dependent action of an aminophos-

pholipid-specific translocase [144, 585, 643, 721]. The loss of phospholipid transbilayer asymmetry in the erythrocyte involves 1) a reorganization of the membrane skeleton, disabling the interaction with plasma membrane phospholipids [104, 217, 349, 423, 703], and 2) an increased transbilayer movement of phospholipids via disturbances of the lipid bilayer structure [39, 218, 219].

It is becoming increasingly evident that platelets, besides a phospholipid transbilayer asymmetry similar to erythrocytes [50, 98, 494, 569, 729], also contain a membrane skeleton [147, 207, 215, 331, 471, 472, 482, 546], functionally similar to that of the erythrocyte [204]. Therefore, the role of calpain-mediated cytoskeletal degradation in the transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity (i.e. the loss of phospholipid transbilayer asymmetry), is thought to be disabling the interaction between membrane skeleton and membrane lipids, thereby enabling the phospholipids to participate in transbilayer movement. However, it may well be that the loss of interaction between membrane skeleton and plasma membrane, by itself, produces local disturbances in the lipid bilayer (flipsites) via which transbilayer movement of phospholipids can proceed at enhanced rates. This suggestion has been put forward earlier [39, 219, 423] and is documented by observations that changes in the organization of the membrane skeleton are accompanied by changes in the structural organization of the lipid bilayer of the plasma membrane, in erythrocytes [15, 95, 103, 194, 526, 703] as well as in platelets [28].

The notion that the action of calpain towards the membrane skeleton can produce disturbances in the organization of the lipid bilayer would explain the observed increase in hydrolysis of membrane phospholipids by phospholipase A₂ when platelet procoagulant activity is increased [50], considering the dependence of phospholipase A₂ activity on the lipid packing [157]. The dual role of calpain-mediated degradation of the membrane skeleton in the loss of phospholipid transbilayer asymmetry, both in enabling aminophospholipids to participate in transbilayer movement and in inducing local disturbances in lipid bilayer organization, is further evidenced by the observation that hydrolysis of the erythrocyte membrane skeleton by calpain leads to increased transbi-

layer movement of phosphatidylethanolamine and phosphatidylserine, accompanied by disturbances of the lipid bilayer [95].

Fast outward transbilayer movement of phospholipids may not be dependent on disturbances of the lipid bilayer (flipsites), since it is recently proposed that the ATP-dependent translocase functions bidirectionally (flippase) [702]. However, to explain fast outward transbilayer movement of phosphatidylserine by the action of a flippase, finished within 2 min after addition of Ca^{2+} to fluoride-treated platelets, one must assume that the ATP requirement of the flippase is fulfilled already at very low ATP levels, considering the prolonged metabolic

inhibition by fluoride. If ATP is not necessarily hydrolysed during flippase action, it may be that ADP/AlF_4^- is able to sustain flippase action, analogous to the activation of G-proteins by GDP/AlF_4^- .

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CHAPTER

4

Phospholipid transbilayer asymmetry in artificially induced platelet plasma membrane vesicles.

ABSTRACT

Incubation of human platelets with unilamellar vesicles composed of dilauroyl-phosphatidylcholine (DLPC) induced shedding of small vesicular structures from the platelet plasma membrane. No significant cell lysis is observed during the process of shedding. Isolated spicules contain the major membrane glycoproteins, I_b, II_b, and III_a, which are used to define the sidedness of the spicule membrane. These glycoproteins are completely susceptible to chymotrypsin treatment, whereas cytoskeletal proteins are inaccessible towards this enzyme. This demonstrates that the spicule membranes have a right-side-out orientation in as far as membrane proteins are concerned. Isolated spicules were 30-fold more active than platelets in stimulating prothrombin conversion to thrombin by the prothrombinase complex (factors X_a, V_a, and Ca²⁺). The increased prothrombinase activity reflects an increased amount of phosphatidylserine in the outer leaflet of the spicule membrane. Protein analysis of platelet spicules and native platelets reveals a number of differences, the most conspicuous of which is the virtual absence of myosin in the spicule preparations. It is proposed that a lack of myosin produces a different cytoskeletal organization in the spicules. This enables phosphatidylserine to become exposed at the outer surface of the spicule membrane.

INTRODUCTION

As reviewed by Op den Kamp [476], asymmetric distribution of phospholipids is a common feature of most, if not all, biological membranes. In the plasma membrane of platelets the negatively charged phospholipids phosphatidylserine (PS) and phosphatidylinositol (PI) are mainly confined to the cytoplasmic leaflet, whereas sphingomyelin (SphM) is predominantly located in the outer half of the membrane. In contrast to the rather extreme distribution of these phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are present on either side of the membrane, though not to the same extent [98, 494, 568, 729].

Upon activation of platelets, in particular by collagen plus thrombin or by the non-physiological Ca^{2+} ionophore A23187, the non-random distribution of phospholipids is partially lost, which results in an increased exposure of PS at the outer surface of the platelet [49, 50]. The increased exposure of PS is reflected by an increased capability of platelets to enhance two sequential reactions of the coagulation cascade, i.e. the activation of factor X by a complex of factors IX_a and VIII_a and the activation of prothrombin by a complex of factors X_a and V_a [49, 50, 543a]. The rate of both coagulation reactions is critically dependent on the presence of a negatively charged phospholipid surface. A close correlation was found between platelet procoagulant activity and exposure of PS at the platelet outer surface [50].

Exposure of PS in the outer leaflet of the platelet plasma membrane presumably results from an increased transbilayer movement of phospholipids (flipflop) [49, 50]. Recent data demonstrated that an increased exposure of PS strictly coincides with an increased breakdown of several cytoskeletal proteins by the action of calpain, an endogenous Ca^{2+} -dependent protease in platelets [124]. From this it was suggested that, in analogy to the erythrocyte membrane [217, 218, 220, 256, 259, 423, 703], interactions between membrane phospholipids and underlying cytoskeletal proteins play an important role in regulation of membrane phospholipid asymmetry.

Recently, Kobayashi and co-workers [330] found that spicules, derived from rabbit platelets upon incubation with dilauroylphospha-

tidylcholine (DLPC) vesicles, were essentially devoid of a 200 kDa protein, representing the heavy chain of myosin, one of the major cytoskeletal proteins in platelets. The aim of the present study was to investigate whether similar spicules could also be derived from human platelets and to see if and to what extent an alteration in composition of cytoskeletal proteins is accompanied by a change in phospholipid distribution in the membranes of these spicules. The ability of the spicules to enhance the activation of prothrombin by a complex of factors X_a and V_a (prothrombinase activity) was used to monitor the exposure of PS in the outer leaflet of the spicule membrane.

MATERIALS AND METHODS

Dilauroylphosphatidylcholine, α -chymotrypsin, fatty acid-free human serum albumin, peroxidase-conjugated concanavalin A and peroxidase-conjugated wheat germ agglutinin were purchased from Sigma Chemical Co. (St. Louis, USA). The prostacyclin analogue ZK 36374 was a kind gift of Schering AG (Berlin, BRD). H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride (S2238), a chromogenic substrate specific for thrombin was from AB Kabi Diagnostica (Stockholm, Sweden). All other reagents were of the highest grade commercially available.

Isolation of platelets.

Washed human platelets were obtained from freshly drawn blood by differential centrifugation as described before [50]. Platelets were finally resuspended in a Hepes buffer composed of 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2 , 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100 nM ZK 36374, 5 mM glucose, pH 7.5.

Preparation of DLPC vesicles.

DLPC was dried from a solution in chloroform/methanol (1/2, v/v) by a stream of nitrogen. Hepes buffer was added and after sonication for 15 min, large particles were removed by centrifugation at 50000 $\times g$ for 30 min. The phospholipid concentration was determined according to Böttger et al. [66].

Incubation of platelets with DLPC vesicles.

One volume of DLPC vesicles was added to nine volumes of a suspension containing $5 \cdot 10^8$ platelets/ml. The final concentration DLPC was 100 μM . Incubation was carried out at 22°C without stirring. To determine the amount of platelet lysis, lactate dehydrogenase activity present in a 30000 $\times g$ supernatant was measured according to Wroblewski and La Due [710].

Isolation and characterization of platelet spicules.

Platelet spicules were isolated by centrifugation of the total platelet/vesicle incubation at 800 $\times$ g for 10 min. The resuspended 800 $\times$ g pellet is further referred to as remnant platelets. The supernatant, which contains spicules, DLPC vesicles and some residual platelets, was centrifuged at 30000 $\times$ g for 30 min to separate spicules from DLPC vesicles. The pellet containing the spicules was resuspended in 1 ml Hepes buffer and layered on top of 15 ml 30% Percoll prepared in the same buffer. After another 30 min centrifugation at 30000 $\times$ g a single band of pure platelet spicules was isolated. Percoll was removed afterwards by three additional washings in Hepes buffer. Prothrombinase activity was measured as described below. Phospholipids analysis was carried out after extraction according to Bligh and Dyer [61] and two-dimensional thin-layer chromatography as described previously [666].

Fatty acid composition of total platelet phospholipids was analyzed by gas-liquid chromatography. From the ratio lauric acid over total fatty acid, the amount of exogenously incorporated DLPC could be calculated. Polyacrylamide gel electrophoresis was performed in the Laemmli system [356], using 7.5% polyacrylamide slab gels with a 4% stacking gel. Samples for electrophoresis were dissolved in 2% SDS (w/v), 5% β -mercaptoethanol (v/v), 50 mM Tris-HCl and 1 mM EDTA, pH 8. Protein determination in the electrophoresis samples were done after 100-fold dilution using the method of Lowry. Gels were stained with Coomassie Brilliant Blue for total protein. Glycoproteins were visualized on nitrocellulose blots using peroxidase-conjugated lectins (wheat germ agglutinin or concanavalin A).

Assay of prothrombinase activity.

Purified coagulation factors Xa, Va, and prothrombin were prepared as described elsewhere [543a, 544]. Prothrombinase activity of either the total incubation mixture of platelets and DLPC vesicles or of the isolated spicules was measured as follows: 312 μ l platelet or spicule suspension was incubated for 3 min in the presence of 13 μ l CaCl_2 (final concentration 3 mM). Subsequently 50 μ l of a mixture of factors Xa and Va (30 and 60 nM, respectively) was added 2 min later, followed by addition of 125 μ l prothrombin (16 μ M in a buffer containing 50 mM Tris, 175 mM NaCl, 14 mM CaCl_2 , and 0.5 mg/ml human serum albumin, pH 7.9). After 1 min, prothrombin activation was arrested by transfer of a 25 μ l subsample into a cuvette containing 1 ml 50 mM Tris, 20 mM EDTA, 175 mM NaCl, pH 7.9. The amount of thrombin formed was calculated from the change in absorbance at 405 nm after addition of chromogenic substrate S2238 to a final concentration of 150 μ M, using a calibration curve made with active site titrated thrombin.

Electron microscopy.

Platelet suspensions were fixed with glutaraldehyde (2.5% in 100 mM phosphate buffer, pH 7.3). The samples were washed and postfixed in osmium tetroxide (1% in the same phosphate buffer). Dehydration was carried out in a graded series of ethanol after which the preparations

were embedded in Epon 812. Ultrathin sections were stained with a saturated solution of uranylacetate and lead citrate (0.25% w/v) adjusted to pH 12 using NaOH. Micrographs were taken with a Philips CM12 microscope. Isolated spicules were negatively stained with 2% uranylacetate on carbon-coated formvar-films.

RESULTS

Addition of DLPC vesicles to a platelet suspension results in a rapid increase of prothrombinase activity, which reaches a maximal value after approximately 45 min incubation (Fig. 1). The increased prothrombinase activity is not the result of exposure of the cytoplasmic side of the membrane due to cell rupture, since less than 3% of the cells were lysed after 60 min incubation. Addition of 100 nM ZK-36374, a stable carbacyclin derivative of prostacyclin, which is a powerful inhibitor of platelet aggregation and release [27], did not prevent expression of procoagulant activity.

Fig. 1. Time dependent prothrombinase activity upon incubation of washed human platelets with DLPC vesicles. Samples were taken from the incubation mixture at different time intervals and diluted with Hepes buffer (pH 7.5) for measuring prothrombinase activity. Platelet concentration in the prothrombinase assay was $1.2 \cdot 10^7 \text{ ml}^{-1}$. Prothrombinase activity is expressed as the rate of thrombin formation (nM per min).

Significant morphological changes accompany the development of procoagulant activity in platelets incubated with DLPC vesicles (Fig. 2). Although it cannot be excluded that the process of spicule formation is not immediately arrested by addition of glutaraldehyde, the appearance of small vesicular structures, pinched off from the platelets, can already be observed one minute after addition of DLPC vesicles. Apart from the formation of more spicules no further morphological changes are observed upon prolonged incubation up to 60 min. Isolation of these platelet spicules on Percoll, followed by negative staining, showed under the electron microscope spherical structures with diameters ranging from 100 to 150 nm.

Fig. 2. Electron micrographs of control platelets (A) and platelets incubated with DLPC vesicles for 1 min (B).

Prothrombinase activities of isolated platelet spicules, control platelets and remnant cells are presented in Table I. The prothrombinase activity of a cell or vesicle preparation depends on the concentration as well as the composition of the phospholipids. To eliminate the effect of different phospholipid concentrations, prothrombinase activities were expressed per μmol phospholipid. Isolated platelet spicules exhibit a prothrombinase activity of 218 nmol thrombin/min per μmol phospholipid, which is a 30-fold increase with respect to the activity of intact control platelets. Remnant platelets have a prothrombinase activity of 52 nmol thrombin/min per μmol phospholipid, which is also increased relative to control platelets though considerably less than observed for platelet spicules. Maximal prothrombinase activity of platelet remnants and spicules can be obtained after disruption of the membranes by sonication. As shown in Table I, the prothrombinase activities for the various sonicated preparations do not differ significantly when expressed per μmol phospholipid.

The phospholipid composition of control platelets, platelet spicules and remnants are similar (Table II), although the amount of PC in the platelet spicules and remnants is increased due to the incorporation of exogenous DLPC (17.3 and 14.9%, respectively). Therefore, it is concluded that the elevated prothrombinase activity of platelet spicules is not the result of an enrichment in procoagulant phospholipids, but must be due to increased exposure of PS at the surface of the spicules. Phospholipases, either alone or in combination with phospholipid transfer protein, have been used successfully to localize phospholipids in the plasma membrane of various cells [217, 476].

TABLE I. Prothrombinase activities of control platelets, platelet spicules, and remnant platelets after incubation with DLPC vesicles.

	Prothrombinase activity	
control platelets	8	(1250)
platelet spicules	219	(1442)
remnant platelets	52	(1012)

Prothrombinase activities are expressed as nM thrombin/min per μmol phospholipid. Values in parenthesis are obtained after complete lysis by extensive sonication.

Fig. 3. SDS-polyacrylamide gel electrophoresis of control platelets, isolated DLPC induced platelet spicules, and remnant platelets after removal of platelet spicules and DLPC vesicles. Gels were stained with Coomassie Brilliant blue. Cytoskeletal proteins are indicated on the left and molecular weight standards on the right. The arrows identify major differences between the protein patterns. Spicules were isolated after 45 min incubation of platelets (5×10^8 per ml) with DLPC vesicles ($100 \mu\text{M}$), as described in Materials and Methods.

Attempts to apply this technique on isolated spicules resulted in virtually complete hydrolysis of all phospholipids and extensive lysis of the spicules as judged from the liberation of lactate dehydrogenase (data not shown). Presumably, the small size of the platelet spicules does not allow the use of these techniques, as was also found for spicules derived from sickle cells [217]. Therefore, the distribution of phospholipids over the spicule membrane remains uncertain.

Protein analysis of isolated spicules and control platelets reveals a number of differences as illustrated in Fig. 3. The most conspicuous difference is the virtual absence in spicules of a band at 200 kDa, corresponding to the heavy chain of myosin. Furthermore, two increased bands at 125 kDa and 98 kDa are observed in the spicules. These bands likely represent increased amounts of glycoproteins II_b and III_a (see also Fig. 4). Finally, the spicules show a distinctly increased band at 76 kDa and lack a band at 59 kDa, both of which have not been identified. Similar protein patterns were obtained by Kobayashi et.al. [330] for spicules isolated from rabbit platelets upon treatment with DLPC.

Advantage was taken of the presence of glycoproteins in the spicules to define the sidedness of the spicule membrane. Glycoproteins were detected on nitrocellulose blots stained with peroxidase-conjugated concanavalin A or wheat germ agglutinin before and after treatment of the spicules with α -chymotrypsin. As a control, intact platelets were treated and analysed in the same manner.

TABLE II. Phospholipid composition of control platelets, platelet spicules and remnant platelets after treatment with DLPC vesicles.

phospholipid class	control platelets	platelet spicules	remnant platelets
phosphatidylserine	10.8 ± 0.5	8.5 ± 0.5 (10.3)	9.2 ± 1.1 (10.9)
phosphatidylinositol	3.1 ± 1.3	1.7 ± 0.4 (2.1)	3.1 ± 1.8 (3.7)
phosphatidylethanolamine	26.8 ± 1.8	19.7 ± 0.9 (23.8)	21.3 ± 1.0 (25.1)
phosphatidylcholine	39.8 ± 1.6	52.1 ± 0.9 (42.1)	48.2 ± 2.0 (39.3)
sphingomyelin	19.3 ± 0.5	18.0 ± 0.8 (21.8)	17.8 ± 0.6 (21.0)

Data are expressed as mol% of total phospholipid fraction (mean $\pm$ S.D., $n=3$). The numbers in parenthesis represent mol% of total phospholipids corrected for incorporated DLPC, which amounts 17.3 and 14.9% in the spicules and remnant platelets, respectively.

Fig. 4A shows the concanavalin A-stained blots of platelets and spicules before and after treatment with chymotrypsin. Complete loss of the concanavalin A-binding sites of glycoproteins II_b and III_a was found after chymotrypsin treatment of both platelets and platelet spicules. A similar finding was obtained upon wheat germ agglutinin staining (Fig. 4B), showing complete removal of the wheat germ agglutinin binding sites of glycoproteins I_a and I_b. Moreover, more than 97% of the sialic acid residues in the spicule preparation are removed upon chymotrypsin treatment. These findings demonstrate that, in comparison with the plasma membrane of control platelets, the spicule membranes are right-side-out with respect to the orientation of the major glycoproteins. In addition, Coomassie-stained gels of control platelets and platelet

spicules before and after chymotrypsin treatment (Fig. 4C) reveal no detectable hydrolysis of the cytoskeletal proteins talin (235 kDa), myosin (200 kDa), α -actinin (105 kDa) and actin (43 kDa), indicating that these proteins are indeed localized in the interior of the spicules. Moreover, this finding indicates that the spicule membrane and platelet plasma membrane remain intact after chymotrypsin treatment, in line with the fact that no lactate dehydrogenase was liberated from the spicules during the incubation. The small, but apparent degradation of filamin (250 kDa) in both intact platelets and spicules upon chymotrypsin treatment is rather unexpected and difficult to reconcile with the cytosolic localization of filamin. It is possible, however, that an un-

Fig. 4. SDS-polyacrylamide gel electrophoresis of control platelets (P) and isolated DLPC-induced platelet spicules (S) before (-) and after (+) treatment with chymotrypsin. (A) Nitrocellulose blot stained with peroxidase-conjugated concanavalin A. (B) Nitrocellulose blot stained with peroxidase-conjugated wheat germ agglutinin. (C) Gel stained with Coomassie Brilliant blue. Major glycoproteins (GP) are indicated in the figure: GPI_a at 150 kDa, GPI_b at 132 kDa, GPII_b at 125 kDa, and GPIII_a at 98 kDa.

known ectoprotein migrates at the same position in the gel as filamin.

Additional information on the protein composition of the spicules comes from comparison of the concanavalin A- and wheat germ agglutinin-stained protein patterns (Figs. 4A and 4B). Platelet spicules seem to be enriched in glycoproteins II_b and III_a, although this could partially be a reflection of the increased ratio of membrane proteins over total protein, due to the small size of the spicules. Furthermore, from the patterns stained with wheat germ agglutinin it appears that the spicules lack a glycoprotein at approximately 160 kDa, which is possibly identical to glycoprotein I_a. Preliminary studies using two-dimensional gel electrophoresis have confirmed the lack of glycoprotein I_a and showed that also glycoprotein II_a is virtually absent in the spicules (Clemetson, K.J., personal communication).

To summarize the differences in protein composition between spicules and remnant platelets, their selective enrichment in cytoskeletal proteins is shown in Fig. 5, in relation to the process of spicule formation.

Fig. 5. Differences in protein composition between DLPC-induced platelet spicules and remnant platelets in relation to the process of spicule formation. Increase or decrease in the content of cytoskeletal proteins relative to control platelets is indicated by upward and downward arrows, respectively.

DISCUSSION

In the present paper we demonstrate that the prothrombinase activity of a suspension of human platelets rapidly increases upon incubation with DLPC vesicles. The increased prothrombinase activity predominantly resides on the surface of small vesicular structures which can be separated from remnant platelets and DLPC vesicles on a Percoll gradient. Since the ability of a membrane surface to enhance prothrombinase activity is determined by the presence of negatively charged phospholipids, in particular PS [50, 543a], it is concluded that spicule formation is accompanied by an increased exposure of PS to the external medium containing the coagulation factors.

The increased exposure of PS on the spicule surface is not due to an inversion process of the platelet plasma membranes, which would lead to exposure of PS present in the cytoplasmic leaflet of the plasma membrane. This is based on the following observations: (1) no appreciable lysis accompanies the process of spicule formation, and (2) spicule membranes are right-side-out, as judged from the orientation of the membrane (sialo-)glycoproteins and the localization of cytoskeletal proteins within the spicules. Furthermore, the increased exposure of PS is not the result of a selective enrichment of this phospholipid, since, apart from the amount of incorporated DLPC, no significant change in composition of the other lipids could be observed in the isolated spicules. The small increase in PS content of the isolated spicules in comparison to control platelets, as reported by Kobayashi et al. [330], might be due to a different isolation procedure of the spicules or merely reflects a species difference (rabbit vs. human platelets). Finally, the increased exposure of PS is not caused by a platelet release reaction, because this is effectively inhibited by the presence of a stable prostacyclin analogue. It is therefore concluded that the shedding process, induced by DLPC, causes a spontaneous loss of the asymmetric distribution of PS over the platelet plasma membrane.

It is presently unknown whether the increased exposure of PS induced by DLPC occurs during the actual shedding process or is the result of a redistribution of this lipid in the spicule membranes, after their detachment from the main cell body. It might be speculated,

however, that the exfoliation of the plasma membrane as such, which precedes the formation of membrane vesicles, is sufficient to cause at least some loss of transbilayer asymmetry of PS, since the remnant cells also exhibit an increased prothrombinase activity.

The rate at which the prothrombinase activity of platelets increases upon incubation with DLPC, suggests a fast redistribution of PS in the platelet or spicule membrane. A rapid loss of transbilayer asymmetry of PS is likely to require both enhancement of transbilayer movement and a loss of stabilizing forces, as for instance provided by cytoskeletal interactions. An increased transbilayer movement could be facilitated by the relatively large amount of incorporated DLPC (approx. 20%) in the membranes. The presence of these short fatty acyl chain phospholipids might be responsible for disturbances of the lipid bilayer structure, at which transbilayer movement of phospholipids is facilitated.

Extensive studies on erythrocytes have led to the concept that the asymmetric distribution of PS in the red cell membrane is maintained by two independent mechanisms, i.e. (1) its ATP dependent translocation towards the inner monolayer [144, 643, 721], and (2) its interaction with the membrane skeleton, in particular with spectrin [217, 256, 259, 703]. Recently, the existence of both mechanisms has been demonstrated in a single study by Middelkoop et.al. [420].

The absence of myosin in the platelet spicules suggests an alteration of the molecular architecture of the cytoskeleton. In analogy to the model which explains the asymmetric distribution of PS in the red cell membrane, we propose that a different cytoskeletal organization in the platelet spicules is responsible for a loss of interaction between PS and cytoskeletal proteins. As a result, PS (partially) randomizes

over both membrane leaflets. The involvement of a membrane cytoskeleton interaction in regulating transbilayer phospholipid asymmetry in the platelet plasma membrane is supported by previous studies [124, 663, 664] in which it was demonstrated that upon stimulation with various agonists, the extent of PS exposure at the outer surface of the platelet is related to the extent of degradation of cytoskeletal proteins (including myosin) by calpain, the endogenous Ca^{2+} -dependent protease. In this respect it is of interest to note that two different myosin isoenzymes have been demonstrated in platelets [492]: a soluble form which represents cytosolic myosin and a slightly different form, which comprises about 25% of the total myosin and appears to be strongly bound to the membrane since it could not be removed by repeated washings.

An asymmetric distribution of phospholipids over the platelet plasma membrane is likely to involve an energy requiring process. It may be speculated that the myosin ATPase activity in platelets could play an active role, either via a direct interaction with amino phospholipids or through maintenance of a particular cytoskeletal conformation, which would be required for an interaction with specific phospholipids. Experiments determining the effect of energy depletion on the localization of PS in the platelet plasma membrane are now in progress in our laboratory.

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CHAPTER

5

Transbilayer asymmetry of membrane fluidity and cholesterol in unstimulated platelets.

ABSTRACT

The existence of a transmembrane fluidity gradient in human platelets was investigated employing trimethylammonium-diphenylhexatriene (TMA-DPH) to probe membrane fluidity and ultrasensitive time-resolved fluorimetry to monitor and analyze the fluorescence lifetime and motional properties of TMA-DPH. Lifetime analysis allowed direct measurement of the amount of TMA-DPH left in aqueous solution. The motional properties of TMA-DPH are represented by steady-state and time-resolved fluorescence anisotropy, and further detailed by structural order parameter and rotational diffusion coefficient. Both by fluorescence intensity and lifetime measurements, it was found that TMA-DPH gradually penetrates the platelet interior, on a time scale of several hours at 37°C. During penetration of TMA-DPH into platelets, fluorescence lifetime, steady-state fluorescence anisotropy, and structural order parameter decreased, and rates of rotational movement increased dramatically. These changes in fluorescence lifetime and motional properties of TMA-DPH during its penetration into unstimulated platelets indicate that the outer leaflet of the plasma membrane is much less fluid than intracellular membranes and the cytosolic leaflet of the plasma membrane. To designate the membrane components responsible for the observed transmembrane fluidity gradient, the average fluorescence lifetime and motional properties of TMA-DPH in various membrane systems were investigated. These membrane systems include: sonicated platelets, platelet lipid vesicles, and phosphatidylcholine vesicles with and without either sphingomyelin, phosphatidylethanolamine, phosphatidylserine or cholesterol as additional component. From these reference membrane systems it is clear that cholesterol is the major determinant of membrane fluidity, as monitored by TMA-DPH. In addition, sphingomyelin, acyl chain unsaturation and membrane proteins also influence membrane fluidity though only moderately as compared to cholesterol. In order to quantitate the contribution of cholesterol to the fluorescence lifetime and motional properties of TMA-DPH in membranes, dose-response relationships were determined. From these relationships, from the properties of TMA-DPH in the outer leaflet of the platelet plasma membrane, and from the relative contributions of the various membrane components to these properties, the cholesterol content of the outer monolayer is determined to yield a cholesterol/phospholipid ratio of 1.0. Assuming a cholesterol/phospholipid ratio of 0.7 for the whole platelet plasma membrane, the cholesterol/phospholipid ratio of the inner leaflet of the plasma membrane would amount to 0.4. The observed transmembrane fluidity gradient is explained in terms of the heterogenous distribution of lipids throughout the human platelet.

INTRODUCTION

The lipids of the platelet plasma membrane are involved in various processes of platelet activation. At the cytosolic side they undergo fast membrane fusion during exocytosis [735], and supply precursors for second messengers like the polyphosphoinositides [397, 461] and esterified arachidonic acid [96, 152]. At the extracellular side, they are responsible for the platelet procoagulant activity, after transbilayer redistribution [54, 733].

In coherence with this functional asymmetry, the lipids of the platelet plasma membrane display a distributional asymmetry: the cytosolic leaflet is enriched with the aminophospholipids (PE, PS), while the cholinephospholipids (PC, SphM) are predominantly present in the outer monolayer of the platelet plasma membrane [98, 494, 569]. Moreover, the acyl-chain composition of phospholipids also differs between the two leaflets of the platelet plasma membrane: arachidonic acid is predominantly present in the cytosolic leaflet [494], fulfilling the needs for prostaglandin synthesis.

A neglected aspect of phospholipid transbilayer asymmetry in the platelet plasma membrane, is the possibility of an associated difference in fluidity between the two bilayer halves [577]. Such a fluidity gradient is found in the erythrocyte [163, 428, 587, 634, 703], the outer leaflet being more rigid than the inner leaflet. Considering the similarity in phospholipid transbilayer asymmetry between platelets and erythrocytes [154, 476, 736], it can be speculated that the inner monolayer of the platelet plasma membrane will be more fluid than the outer monolayer. This would also be predicted from the rigidifying effect of SphM [62], and the fluidizing effect of unsaturated acyl-chains on lipid order [621].

The possibility of a fluidity gradient across the platelet plasma membrane was investigated

employing the fluorescent membrane probe TMA-DPH. This probe seemed most suitable for studying transbilayer asymmetry of membrane fluidity, because of its particular properties. Addition of TMA-DPH to a cell suspension results in immediate labeling of the outer monolayer of the plasma membrane [345, 348]. However, most of TMA-DPH will remain in aqueous solution, due to a partition equilibrium [347], where it is hardly fluorescent [519]. Transbilayer movement of TMA-DPH [137] will result in a gradual labeling of intracellular compartments [347, 348]. Thus, the rationale has been that a fluidity gradient in the platelet plasma membrane would be revealed by a time-dependent change in dynamic fluorescence properties of TMA-DPH. An important advantage of this approach is the use of intact cells, since disruption of the interaction between cytoskeleton and membrane could result in loss of transmembrane fluidity gradient [634, 703]. In addition, TMA-DPH is not cytotoxic [345, 347], allowing measurements in viable cells for prolonged periods.

Ultrasensitive time-resolved fluorescence depolarization was applied to monitor the changes in fluorescence properties of TMA-DPH. This technique has proven its versatility in lipid bilayers [47, 320] and biomembranes [12, 32, 286, 320, 487, 620], and provides a more detailed description of motional characteristics than fluorescence depolarization. Simultaneously, information about the fluorescence lifetime of TMA-DPH is obtained, which is an indicator for the polarity of the environment of TMA-DPH [519, 617, 618]. Interpretation of fluorescence anisotropy decays is dependent on the model describing the motion of the probe in the membrane [320]. In this respect, the use of TMA-DPH has an additional advantage: owing to its unimodal distribution in lipid bilayers parallel to the acyl-chains [617], its motional properties are adequately described by several diffusion models, like the general diffusion model [9] and the "wobbling-in-cone" model [9, 322]. The use of time-resolved fluorescence depolarization raises the opportunity to discriminate between discrete probe populations [487, 670], and thus allows determination of the relative amount of TMA-DPH in aqueous solution.

Using the methodological approach outlined above, it will be shown that gradual penetration of human platelets by TMA-DPH is

ABBREVIATIONS

PE: phosphatidylethanolamine, PS: phosphatidylserine, PC: phosphatidylcholine, SphM: sphingomyelin, TMA-DPH: trimethylammonium-diphenylhexatriene, C/P ratio: cholesterol/phospholipid ratio, F: fluorescence intensity, TNP-Gly: trinitrophenyl-glycine, TNP-Glu: trinitrophenyl-glutamate, TNP-Lys: trinitrophenyl-lysine.

accompanied by dramatic changes in fluorescence properties of TMA-DPH, indicative of a significant difference in fluidity between the outer and inner monolayer of the platelet plasma membrane. In an attempt to designate the factor(s) responsible for the fluidity gradient, the effect of the major components of the platelet plasma membrane on the fluorescence properties of TMA-DPH in model systems was determined. From these results it is concluded that the transbilayer asymmetry of membrane fluidity can not only be explained by differences in acyl-chain unsaturation and SphM content, but also implicates an asymmetric transbilayer distribution of cholesterol. Based on a detailed investigation of the effects of cholesterol on the fluorescence properties of TMA-DPH and data from literature, it is proposed that the outer and inner leaflet of the platelet plasma membrane have a C/P ratio of 1.0 and 0.4, respectively. The observed transmembrane fluidity gradient will be discussed in terms of the heterogeneous distribution of lipids throughout the human platelet, and its physiological implications will be addressed.

MATERIALS AND METHODS

TMA-DPH was from Molecular Probes Inc. (Plano TX, USA). Cholesterol, PC and PE from egg-yolk, and SphM and PS from bovine brain were from Sigma (St. Louis MO, USA). All lipids were used without further purification, except for PS, which was further purified by CM-cellulose column chromatography as described [126]. Purity of lipids was confirmed by thin-layer chromatography. All other chemicals were of the highest grade commercially available.

platelets

Human platelets were isolated, as outlined previously [51], by differential centrifugation from fresh blood drawn from healthy volunteers who received no medication. Blood was anticoagulated with acid-citrate-dextrose. Platelets were washed in an isotonic buffer at pH 6.6, containing 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM glucose (Hepes buffer) in the presence of 0.05% fatty acid-free human serum albumin. They were finally resuspended at a concentration of 2×10^8 per ml in Hepes buffer pH 7.4 without albumin. Platelet concentration was inferred from their absorption at 405 nm using a calibration curve determined with a Coulter Counter. Platelet lysis was determined by assaying lactate dehydrogenase (EC 1.1.1.27) in a 12000g supernatant according to Wroblewski and La Due [710].

Total platelet membranes were prepared by sonication in the presence of 1 mM EGTA for 5 min, with 10 sec intervals, in a direct-probe sonicator (MSE) at an amplitude of 12 μ m. Total platelet lipids were extracted according to Bligh and Dyer [61], from platelets pooled from 8 donors.

Vesicles

Small unilamellar vesicles were prepared by sonication of hydrated lipids. Lipids were stored in chloroform/methanol (1/1, by vol.) at $-20^\circ C$ under N_2 . Aliquots of lipid solutions were evaporated to dryness under a stream of N_2 . Dry lipids were hydrated by addition of Hepes buffer (pH 7.4, without albumin), which was degassed with N_2 . Vesicles were prepared by sonication for 5 min above their transition temperature, with an amplitude of 14 μ m. Cholesterol containing vesicles were sonicated for 10 min. After sonication vesicles were centrifuged at 14,000g for 5 min. The final phospholipid concentration was determined according to Böttcher et al. [66], and varied between 2 and 5 mM. Cholesterol concentration was determined enzymatically by the cholesterol-oxidase assay, using a commercial kit (Sigma, St. Louis MO, USA).

Fluorescence measurements

TMA-DPH was dissolved in N,N-dimethylformamide at a concentration of 5 mM and stored at $-20^\circ C$ in the dark. For every experiment an aliquot from this stock solution was diluted 10 times in Hepes buffer pH 7.4 to obtain a temporary stock solution, which was found to be stable for at least 5 hours. Washed human platelets were diluted into a 3 ml quartz cuvette with Hepes buffer pH 7.4 to a concentration of 2×10^7 per ml and labeled at $37^\circ C$ with 1 μ M TMA-DPH, added from the diluted stock solution under continuous stirring. Using this double dilution procedure, the final concentration of N,N-dimethylformamide in the cuvette could be kept very low (0.02 vol%). After addition of TMA-DPH, platelets were kept in the dark at $37^\circ C$ without stirring, and at regular intervals fluorescence properties of TMA-DPH were measured. Sonicated vesicles were employed at a concentration between 20 and 50 μ M, under the same conditions as platelets. Preparation of TNP-derivatives of Lysine and Glutamate was based on the procedure of Snyder and Sobocinsky [606], and was performed by reaction of 100 mM trinitrobenzenesulfonic acid with 110 mM lysine or glutamate at pH 9.5 in a mixture of water and dimethylsulfoxide (2/1 by vol.). Both TNP-derivatives exhibited an identical absorption maximum at 420 nm.

Fluorescence intensities (F) were recorded at $37^\circ C$ under continuous stirring (300 rev/min) with an SLM Aminco SPF-500C fluorimeter equipped with a light-tight injection port and a stirring device (Hellma). Excitation and emission wavelengths were set at 365 and 450 nm with 5 and 20 nm slits, respectively. F was corrected for light-scatter and background fluorescence using unlabeled controls.

Phase fluorimetry was performed on a SLM-4800 fluorimeter according to Prendergast et al. [519], using a POPOP reference solution in order to remove any color-dependent response of the detector [361]. Lifetimes from phase shifts (τ_p) were determined at a frequency of 30

MHz, by a crosscorrelation method using the computer program supplied by the manufacturer. Excitation was accomplished using a monochromator set at 360 nm (0.5 nm bandpass). Emission was passed through two KV408 cut-off filters (Schott) before detection. Background fluorescence and light-scatter amounted to less than 1% of total intensity and were neglected. All measurements were performed at 37°C without stirring.

Fluorescence decay and fluorescence anisotropy decay were determined with a laser-pulsed single photon counting fluorimeter, described in detail elsewhere [284, 285, 286, 671]. TMA-DPH was excited at 310 nm by a frequency-doubled, synchronously pumped rhodamine 6G dye laser. Emission was monitored above 450 nm through a K45 band-pass filter (Balzers). All measurements were performed at 37°C without stirring.

Analysis of time-resolved data

Data were analyzed by a reference convolution method described in detail by Vos et al. [673]. Corrections for light-scatter and background fluorescence were routinely made by subtraction of the recording of a control sample without TMA-DPH.

Total fluorescence, $s(t)$, was reconstructed from the parallel, $I_{||}(t)$, and perpendicular, $I_{\perp}(t)$, polarized intensity components to yield the fluorescence lifetime components τ_i with fractional amplitude α_i :

$$s(t) = I_{||}(t) + I_{\perp}(t) = \sum \alpha_i e^{-t/\tau_i}$$

Routinely a biexponential function was employed, generally used by others to describe the fluorescence decays of TMA-DPH [47, 364, 617, 620]. In addition, a tri- or tetra-exponential function, fitting from lower channel numbers, was used to include the fluorescence lifetime of TMA-DPH in solution (fixed at 38 psec). The average fluorescence lifetime, $\langle \tau \rangle$, was calculated from the individual components from:

$$\langle \tau \rangle = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i$$

The anisotropy decay function, $r(t)$, was obtained by simultaneous fitting of $I_{||}(t)$ and $I_{\perp}(t)$ according to:

$$I_{||}(t) = s(t)/3[1 + 2r(t)]$$

$$I_{\perp}(t) = s(t)/3[1 - r(t)]$$

The anisotropy decay of TMA-DPH in membranes can be adequately interpreted within the framework of the "wobbling-in-cone" model [9, 620], introduced by Kinoshita et al. [320, 322]:

$$r(t) = r_{\infty} + (r_0 - r_{\infty})e^{-t/\phi}$$

where r_0 is the fundamental anisotropy, r_{∞} is the residual anisotropy at the end of the experiment (about 30 ns), and ϕ is the apparent relaxation time.

It is generally recognized that "fluidity" is not a well-defined parameter at the molecular level: fluidity in membranes is an anisotropic property, determined by both static (range of movement) and dynamic (rate of mo-

tion) components. Therefore, both components were derived separately. The static component is expressed by the second-rank order parameter, S , and obtained from:

$$S = \sqrt{r_{\infty}/r_0}$$

For comparison with various literature values, the cone-angle, θ_0 , describing the range of acyl-chain motion in the "wobbling-in-cone" model, is determined from:

$$S = 1/2 \cos \theta_0 (1 + \cos \theta_0)$$

The dynamic component is expressed by the model-independent diffusion constant for rotation around an axis perpendicular to the probe symmetry axis, $D_{\perp}$, and is evaluated from:

$$D_{\perp} = (r_0 - r_{\infty})/(6r_0\phi)$$

assuming that the anisotropy decays exponentially to r [9].

To accommodate the frequent use of steady-state anisotropy, r_s , to describe changes in fluidity, r_s was also derived using the Perrin-equation:

$$r_s = r_{\infty} + (r_0 - r_{\infty})/(1 + \tau/\phi)$$

in which τ was substituted by the average fluorescence lifetime, $\langle \tau \rangle$, from a biexponential fit, which excludes any contribution of the ultrafast fluorescence of TMA-DPH in buffer. In addition, steady-state anisotropy can be expressed as the degree of polarization, P , related to r_s :

$$P = 3r_s/(2 + r_s)$$

To eliminate the inaccuracy introduced by variations in r_0 , r_0 was substituted by the average- r_0 , which was determined from 96 experiments and which amounted to 0.318 ± 0.008 within a 1% confidence interval. This value is lower than reported for glycerol as solvent (about 0.39, [47, 519]), as observed before [620], and is similar as found by van Langen et al. [364]. Note, however, that the experiments presented used excitation at 310 nm, which was the highest wavelength possible in the current setup.

RESULTS

Gradual penetration of TMA-DPH into unstimulated platelets.

Of first concern was to show that TMA-DPH, if continuously present, would indeed penetrate the platelet interior and on which time scale this process would take place. Since cellular penetration by TMA-DPH already has been suggested from fluorescence intensity (F)

measurements [347, 348], the development of F was monitored during prolonged incubation of washed human platelets with TMA-DPH. At the conditions employed, the majority of TMA-DPH is still in the aqueous solution ($1 \mu\text{M}$ TMA-DPH and $2 \cdot 10^7$ platelets per ml; vide infra). Under these conditions F was linear with platelet concentration up to 10^8 per ml. After the addition of TMA-DPH, F sharply increased and stabilized within 2 min. At this moment only a fraction of all membranes is labeled, as will be shown later. Taking into account the rapid labeling kinetics, it appears that the outer monolayer is quickly saturated, in agreement with previous reports [345, 348]. Thus, any further change in F would indicate unlabeled membrane becoming available for labeling by TMA-DPH.

Incubation of unstimulated platelets with TMA-DPH showed that F slowly increased with a gradually declining slope (Fig. 1A). After 5 hours at 37°C , a plateau level still was not reached, while F was doubled with respect to the starting value. At this time platelet lysis was 6%, measured as LDH leakage. Therefore, the increase in F cannot be due to platelet lysis. It is tempting to interpret the gradual increase in F as penetration of TMA-DPH into the platelet interior. However, one can imagine that during prolonged incubation of platelets with TMA-DPH, a reorganization of the probe in the bilayer could give rise to increased incorporation of TMA-DPH. Alternatively, a slowly increasing interaction of TMA-DPH with hydrophobic domains of proteins, as illustrated by albumin [166], could also be responsible for the observed increase in F. To explore these possibilities, sonicated platelets were also incubated with $1 \mu\text{M}$ TMA-DPH at 37°C (Fig. 1A). It was found that F gradually decreased to reach a plateau level after 3 to 4 hours. Therefore, it is unlikely that a reorganization of TMA-DPH, without crossing the plasma membrane, is responsible for the observed increase in F during prolonged incubation of platelets with TMA-DPH.

Comparison of F from sonicated platelets and intact platelets, a few minutes after labeling, suggests that F in intact platelets could rise less than a factor of three after disruption. This could be taken to indicate that more than one-third of total platelet lipids is present in the outer monolayer of the platelet plasma membrane. This value apparently deviates from reported estimates of about 25% [50, 98, 494]. It

should be emphasized, however, that quantitative interpretation of F is hampered by underlying changes in fluorescence lifetime, which will be described in a later section.

To obtain a more direct answer as to whether TMA-DPH penetrates unstimulated platelets, advantage was taken of a special property of TMA-DPH: the fast equilibrium between aqueous and lipid phase. At various time intervals during incubation of platelets with TMA-DPH, a subsample was diluted hundredfold into buffer devoid of TMA-DPH under continuous monitoring of F. TMA-DPH present in the outer monolayer of the plasma membrane is expected to reequilibrate rapidly with the buffer, while TMA-DPH within the platelet will reequilibrate much more slowly. It was found that F decreased abruptly upon dilution to reach a stable level within 30 sec, indicative for a new steady-state. It appeared that, during incubation of intact platelets with TMA-DPH for 4 hours, the residual F after dilution increased tenfold (Fig. 1B), indicative for increased uptake of TMA-DPH by platelets. To exclude that increased binding to proteins in the outer

Fig. 1. Gradual penetration of TMA-DPH into unstimulated human platelets, as determined by fluorescence intensity measurements.

(A) Time-dependent changes in fluorescence intensity of $1 \mu\text{M}$ TMA-DPH incubated with unstimulated platelets (●) or sonicated platelets (○) at a concentration of $2 \cdot 10^7$ per ml. (B) Residual fluorescence intensity of TMA-DPH in unstimulated platelets (●) or sonicated platelets (○) after hundredfold dilution in HEPES buffer, pH 7.4. In this case, intact or sonicated washed human platelets were labeled with $5 \mu\text{M}$ TMA-DPH at a concentration of $2 \cdot 10^8$ per ml, before dilution. (A) and (B): both platelet preparations represent equal amounts of platelets. All manipulations were carried out at 37°C . Further details are described in the methods. Data in both figures were obtained simultaneously during a representative experiment.

monolayer of the plasma membrane is responsible for the increase in residual F after dilution, sonicated platelets were used. It was found that during a period of 4 hours, sonicated platelets displayed a constant residual F after dilu-

tion (Fig. 1B). Uptake of TMA-DPH could also result from endocytosis. However, the increase in residual F after dilution is accompanied by an increase in F , which cannot be explained by endocytosis per se. Therefore, it is concluded that TMA-DPH gradually penetrates platelet intracellular compartments as a result of slow transbilayer movement.

Application of time-resolved fluorescence to monitor anisotropy and fluorescence lifetime of TMA-DPH.

The fluorescence anisotropy decay of TMA-DPH in unstimulated platelets (Fig. 2A), as well as in all model membrane systems em-

Fig. 2. Anisotropy decay (A) and intensity decay (B) analysis of TMA-DPH in unstimulated platelets at different incubation times (min). Data shown are representative for 5 separate measurements. The quality of the fit is indicated by the weighted residuals and the autocorrelation function of the weighted residuals on top of the curves. The corresponding fit parameters and derived fluorescence lifetime and anisotropy data are presented in table I and II, respectively. (A) To aid comparison, the noisy experimental anisotropy curves were discarded. The initial anisotropies of the fitted curves were lower than the true ones of ca. 0.31, because the finite pulse width of the instrument leads to suppression of this value [486]. (B) Shown are the results of the tetra-exponential fit, found necessary when analysis was carried out right from the emission peak. (C) Fluorescence decay of $1 \mu\text{M}$ TMA-DPH at 37°C in Hepes buffer pH 7.4 (curve shifted slightly to the right), compared with the excitation pulse. The fluorescence lifetime was 38 ± 3 ps (mean $\pm$ S.D. of three separate determinations). Experimental details are described in the methods.

played, could be adequately described by a single exponential and a residual anisotropy (Table I). This means that the motion of TMA-DPH in lipid bilayers can be adequately described by the "wobbling-in-cone" model [320], in agreement with previous findings [9, 620].

The fluorescence decay of TMA-DPH in platelets (Fig. 2B) as well as in model systems could be well represented by a short (τ_1) and a long (τ_2) lifetime component (Table II), in agreement with other observations [47, 137, 155, 364, 617, 618, 620]. These two components are derived from a biexponential fit starting about 0.5 ns after the emission peak. Because most of TMA-DPH still must be present in aqueous solution, the fluorescence decay of TMA-DPH was measured in Hepes buffer only (Fig. 2C). The fluorescence decay could be described by a single lifetime of about 38 ps. The very short fluorescence lifetime of TMA-DPH in aqueous solution is in agreement with observations of Prendergast et.al. [519]. To incorporate the soluble pool of TMA-DPH in the fluorescence lifetime analysis of TMA-DPH in membrane systems, the fluorescence decay was described by a triexponential function fitting right from the emission peak with τ_1 fixed at 38 ps (Table II). However, this approach did not result in good fits for all membrane systems, especially when the contribution of τ_2 increased (note the statistical criteria listed in Table II). Apparently, including short fluorescence lifetimes in the fit, an additional component, normally not seen by biexponential fits, is uncovered. Hence, the fluorescence decay of

TMA-DPH was analysed by a tetraexponential function, again with τ_1 fixed at 38 ps. This procedure resulted in excellent fits for all membrane systems (Table II). It is noted that the contribution of TMA-DPH in aqueous solution (α_1) is not significantly altered in the tetraexponential fit, supporting the view that the fluorescence decay of TMA-DPH in membranes actually is described by three components, the shortest of which escapes the normal biexponential fits starting some time after the emission peak. The average fluorescence lifetime of TMA-DPH in membranes will be represented by $\langle\tau\rangle$, in accordance with the usual presentation.

Changes in fluorescence lifetime and anisotropy of TMA-DPH during penetration into unstimulated platelets.

Using the sensitivity of time-resolved fluorescence spectroscopy, it becomes possible to determine directly the fraction of TMA-DPH in solution. This is a great advantage, because it allows a full description of the distribution of TMA-DPH in lipid suspensions. This property was employed to reconsider the gradual penetration of TMA-DPH into unstimulated platelets. It was found that the fraction of TMA-DPH bound to platelets gradually increased on a time scale of several hours (Fig. 3), in agreement with the results from fluorescence intensity measurements. After 3 hours, the fraction of TMA-DPH bound to platelets was increased fourfold relative to the bound fraction immediately after labeling. This increase is significantly higher than obtained from fluorescence intensity measurements, which showed a less than twofold increase (Fig. 1A). This difference can be explained in terms of a decrease in fluorescence lifetime of TMA-DPH accompanying the increased penetration into platelets. In this way, F is an underestimation of the bound fraction of TMA-DPH. In view of the amount of phospholipids found to be present in the outer monolayer of unstimulated platelets (approx. 25%) [50, 98, 494], an increase in bound fraction of four times relative to the bound fraction of TMA-DPH immediately after labeling, would be expected when platelets are fully equilibrated with TMA-DPH. Taking into account that

Fig. 3. Gradual penetration of TMA-DPH into unstimulated platelets, as determined from time-resolved fluorescence decay. The bound fraction is equivalent to the sum of the fractional amplitudes α_2 , α_3 , and α_4 from the tetraexponential decay fit, assuming only one water-soluble pool (α_1) described by τ_1 (38 ps). Experimental details are described in the methods. Data shown are pooled from 5 separate experiments.

TABLE I. Fluorescence anisotropy decay parameters of TMA-DPH in various membranes.

membrane preparation	r_0	r	ϕ (ns)	χ^2	DW	Zpar	Zper	r_s	S	P	θ_c (°)	D $\perp$ (MHz)
platelets (t = 0 min)	.321 .011	.249 .001	1.08 .14	1.08	1.88	218	259	.258	.884	.343	22.9	34
platelets (t = 140 min)	.323 .018	.187 .001	0.79 .07	1.14	1.89	245	192	.206	.767	.280	33.3	87
sonicated platelets	.329 .006	.222 .001	1.10 .07	1.05	1.88	181	200	.241	.836	.322	27.6	46
platelet lipid	.326 .005	.203 .001	1.15 .05	1.09	1.85	205	172	.227	.800	.306	30.7	52
PC	.311 .004	.112 .001	1.80 .06	1.11	1.91	202	208	.196	.593	.267	45.7	60
PC/cholesterol C/P = 1.4	.301 .003	.246 .001	2.16 .19	0.99	1.92	214	228	.264	.879	.350	23.5	18
PC/PE (7/3)	.289 .005	.112 .002	2.36 .13	1.25	2.00	236	217	.212	.593	.288	45.7	46
PC/SphM (7/3)	.314 .004	.136 .001	1.85 .06	1.17	1.79	158	224	.202	.653	.275	41.6	52

Fluorescence anisotropy decay was analysed with a single exponential and a residual anisotropy, starting at channel 54 (emission peak at channel 26, total number of channels is 1024, time-equivalence per channel was 32.5 ps). Standard deviations of fitted parameters are indicated underneath. Fit quality can be judged from χ^2 , the Durbin-Watson parameter (DW), and the number of zero passages in the autocorrelation function in the parallel (Zpar) and perpendicular (Zper) component. See also Fig.5. for graphical representation of the most relevant calculated parameters. All measurements were carried out at 37°C as described in the methods. Data shown are representative for at least 3 independent determinations.

Table III. Effect of negatively and positively charged hydrophilic quenchers on the fluorescence lifetime of TMA-DPH in lipid vesicles.

Membrane preparation	quencher	before quencher	after quencher
PC-vesicles	TNP-Lys	2.70 (.04)	1.53 (.03)
	TNP-Glu	2.72 (.05)	2.01 (.04)
PC/PS-vesicles 7/3 molar ratio	TNP-Lys	2.83 (.04)	2.25 (.03)
	TNP-Glu	2.78 (.06)	2.61 (.03)

Quenching of TMA-DPH is monitored by a decrease in fluorescence lifetime as determined by phase fluorimetry (τ_p in ns). Quenchers employed were the neutrally and negatively charged trinitrophenyl derivatives of lysine and glutamate, respectively. Vesicle concentration was 50 μ M phospholipid. Quencher concentration was 100 μ M. Measurements were performed at 37°C as described in the methods. Data shown are the mean ($\pm$ s.d.) of 3 separate determinations.

millimolar Ca^{2+} can decrease membrane binding of TMA-DPH (next chapter), and that the outer monolayer of the plasma membrane is more rigid than the inner (vide infra) and thus may limit uptake of TMA-DPH [720a], the ratio of TMA-DPH vs lipid may be lower in the outer monolayer than in intracellular membranes, resulting in a higher increase in membrane bound TMA-DPH upon penetration into platelets than can be expected on the basis of lipid ratios.

When fluorescence properties of TMA-DPH in unstimulated platelets are examined a few minutes after addition of TMA-DPH (Table I and II, $t=0$ min), an average fluorescence lifetime of 6.8 ± 0.1 ns (biexponential fit) and a steady-state anisotropy of 0.260 ± 0.005 are observed (mean $\pm$ s.d., $n=5$). The fluores-

cence lifetime for TMA-DPH in platelets is similar as reported by Kubina et.al. [345]. The anisotropy is close to that reported by Kubina et.al. [345] and Donner & Stolz [166], who found values of 0.275 and 0.267, respectively. The tetraexponential fluorescence decay (Table I) shows that immediately after addition of $1 \mu\text{M}$ TMA-DPH to $2 \cdot 10^7$ platelets per ml, about 96% of total TMA-DPH is still present in the buffer. Assuming that under these conditions total platelet phospholipids amount to $8 \mu\text{M}$, and that 25% of these lipids are in the outer monolayer, it can be estimated that the TMA-DPH/phospholipid ratio in the outer monolayer is about 1/50.

Using a membrane probe at high probe-lipid ratios, there is a chance that the observed

Fig. 4. Changes in fluorescence lifetime and motional properties of TMA-DPH accompanying its gradual penetration into unstimulated platelets. Presented are the average fluorescence lifetime, $\langle \tau \rangle$, the steady-state anisotropy, r_s , the structural order parameter, S , and the rotational diffusion constant, $D_{\perp}$. All parameters are plotted versus the fraction of TMA-DPH bound to unstimulated platelets at 37°C . Experimental details are described in the methods. Data shown are pooled from 5 separate experiments.

TABLE II. Fluorescence decay parameters of TMA-DPH in various membranes.

membrane preparation	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	α_4	τ_4 (ns)	$\langle \tau \rangle$ (ns)	χ^2	DW	ZP
platelets t = 0 min	.238 .004	2.39 .08	.762 .005	7.35 .02					6.89	1.15	1.88	231
platelets t = 140 min	.475 .005	2.24 .03	.525 .006	5.54 .02					4.66	1.21	1.71	158
sonicated platelets	.388 .003	2.23 .03	.612 .004	6.14 .01					5.41	1.21	1.69	170
platelet lipid	.382 .004	2.32 .03	.618 .005	5.69 .01					5.01	1.19	1.78	185
PC	.360 .005	1.22 .03	.640 .006	2.95 .01					2.62	1.15	1.90	211
PC/cholesterol C/P = 1.4	.209 .007	3.08 .10	.791 .009	6.94 .02					6.53	0.98	1.95	234
PC/PE (7/3)	.439 .007	1.08 .03	.561 .008	2.89 .01					2.48	1.15	2.15	269
PC/SphM (7/3)	.401 .004	1.60 .03	.599 .006	3.72 .01					3.25	1.27	1.73	158
platelets t = 0 min	.958 .003	.038	.009 .001	2.24 .06	.032 .001	7.31 .02			6.02	2.37	1.29	223
platelets t = 140 min	.843 .006	.038	.071 .001	1.83 .02	.086 .001	5.38 .01			4.37	1.61	1.36	28
platelets t = 0 min	.964 .004	.038	.004 .001	1.35 .21	.008 .001	4.15 .50	.024 .001	7.59 .09	5.96	1.18	1.95	267
platelets t = 140 min	.841 .010	.038	.036 .001	0.81 .04	.065 .002	3.07 .09	.058 .002	5.85 .05	4.37	1.08	1.97	251
sonicated platelets	.817 .006	.038	.031 .002	0.99 .07	.062 .002	3.25 .14	.090 .003	6.41 .05	5.21	1.12	1.85	245
platelet lipid	.504 .014	.038	.078 .004	0.81 .05	.184 .006	3.19 .11	.235 .009	5.94 .05	4.95	1.10	1.96	276
PC	.784 .009	.038	.065 .001	0.65 .01	.110 .007	2.43 .05	.041 .007	3.43 .08	2.42	1.11	1.90	233
PC/cholesterol C/P = 1.4	.245 .029	.038	.043 .006	0.79 .14	.178 .016	3.74 .26	.565 .020	7.04 .06	6.51	1.00	1.94	243
PC/PE (7/3)	.393 .030	.038	.156 .007	0.33 .02	.193 .005	1.27 .05	.258 .006	2.93 .02	2.39	1.14	2.15	263
PC/SphM (7/3)	.832 .005	.038	.047 .001	0.80 .02	.088 .005	2.93 .07	.033 .006	4.35 .10	2.99	1.07	2.06	274

Biexponential analysis (upper part) of fluorescence decay was performed from channel 54 (emission peak at channel 26, time equivalence per channel was 32.5 ps). Tri- and tetraexponential analysis (middle and lower part, respectively) started right at the emission peak, with τ_1 fixed at 38 ps. Fit quality can be judged from χ^2 , the Durbin-Watson parameter (DW), and the number of zero passages in the autocorrelation function (ZP). Standard deviations of fitted parameters are indicated underneath. See also Fig. 5. for graphical representation of the most relevant calculated parameters. All measurements were carried out at 37°C as described in the methods. Data shown are representative for 3 or more independent determinations.

dynamic properties are influenced by probe-probe interactions, or that the structural properties of the membrane are disturbed by the presence of the probe in the bilayer. To ensure that the observed anisotropies are not influenced by TMA-DPH itself, the dependence of the anisotropy on the concentration of TMA-DPH was determined. It was found that increasing the TMA-DPH concentration to 2 μM did not affect the observed anisotropies (0.260 – 0.261), while at 5 μM TMA-DPH the anisotropy was significantly reduced (0.260 – 0.253). Therefore, it is concluded that under the conditions employed the observed anisotropies of TMA-DPH are representative for the structural and dynamic state of its lipid environment.

Of major interest was the question what kind of lipid environments TMA-DPH would experience during its movement from the outer monolayer of the platelet plasma membrane to the intracellular membranes. For this purpose the fluorescence lifetime and the steady-state anisotropy of TMA-DPH were determined as a function of the bound fraction of TMA-DPH, which proved a better representation of the level of penetration than the incubation time with TMA-DPH. It was found that, during progressive penetration of TMA-DPH into unstimulated platelets, the average fluorescence lifetime ($\langle\tau\rangle$) and the steady-state anisotropy (r_s) decreased dramatically (Fig. 4). $\langle\tau\rangle$ decreased from 6.9 to 4.5 ns, while r_s dropped from 0.260 to 0.210. Both parameters seem to level off at about 5 to 6 times the original bound fraction of TMA-DPH, and follow a similar time course as the penetration of TMA-DPH (Figs. 1 and 3). Thus, intracellular lipid compartments are much more fluid than the outer monolayer of the plasma membrane.

To discriminate between static and dynamic components of lipid fluidity, the structural order parameter (S) and the rotational diffusion constant ($D_{\perp}$) are also presented as a function of the bound fraction of TMA-DPH (Fig. 4). S decreased dramatically during penetration of TMA-DPH, in a manner identical to that of $\langle\tau\rangle$, from 0.88 to 0.77. Such a close relationship between S and $\langle\tau\rangle$ was also observed by Straume and Litman [617, 618]. They suggested that a decrease in order will be accompanied by an increased penetration of water into the lipid bilayer. The resulting increase in polarity will reduce the fluorescence

lifetime of TMA-DPH. Examination of all the data, irrespective of the type of membrane, confirmed this close relationship between S and $\langle\tau\rangle$. The behavior of $D_{\perp}$ during penetration of TMA-DPH into platelets clearly deviates from the behavior of S : a fast increase is observed, from 10 to about 80 MHz, followed by a relatively stable plateau, which is already reached when the bound fraction of TMA-DPH is doubled. As such, the observation that an increase in rate of rotational motion ($D_{\perp}$) accompanies an decrease in order (S) is in line with observations employing DPH [320] or TMA-DPH [620]. However, the observation that $D_{\perp}$ reaches a plateau while S further decreases may reveal some interesting details about the environment sensed by TMA-DPH in the platelet interior (vide infra).

Characterization of membrane components determining the behavior of TMA-DPH in membranes.

First of all, some evidence will be provided for the location of TMA-DPH in the lipid bilayer, since this is crucial to develop a detailed interpretation of changes in fluorescence lifetime and anisotropy. TMA-DPH was originally synthesized to obtain a membrane probe with a more defined distribution in the bilayer than DPH [519]. Subsequently, this assumption was confirmed by several authors showing that TMA-DPH was unimodally distributed in lipid bilayers [182, 617]. However, a more precise description of the location of TMA-DPH in the lipid bilayers has not been obtained, though it can be expected that TMA-DPH will locate its positive charge at the aqueous boundary of a membrane. Quenching experiments indeed showed that TMA-DPH must be located close to the surface of the membrane [137]. Based on the successful application of TNP-Gly to quench DPH [577], and taking advantage of the positive charge of TMA-DPH, the location of TMA-DPH in the lipid bilayer was investigated with the aid of TNP-Lys and TNP-Glu, which are neutrally and negatively charged, respectively. The differential effects of both quenchers on the average fluorescence lifetime of TMA-DPH, should allow conclusions about its exact location. Fluorescence lifetimes of TMA-DPH in

zwitterionic PC vesicles and negatively charged PC/PS vesicles, in the presence and absence of TNP-Lys or TNP-Glu, were determined by phase fluorimetry. It was found that TNP-Lys was more effective than TNP-Glu in quenching TMA-DPH in PC vesicles (Table III). This is a surprising result, since TNP-Glu, being negatively charged, was expected to quench the positively charged TMA-DPH better than TNP-Lys. These results can only be explained, taking into account the zwitterionic character of PC. TNP-Glu would not reach the phosphate layer but rather stay close to the positively charged choline headgroup, while TNP-Lys would be located close to the negatively charged phosphate group of PC. In line with this reasoning, it is concluded that the ternary amino-group of TMA-DPH is in close proximity of the negatively charged phosphate-group of PC. This conclusion is further confirmed by the effect of addition of PS to PC vesicles (Table III). Applying a negative surface charge, it can be expected that the binding of TNP-Lys to the membrane surface is greatly enhanced. This does not, however, result in more quenching of TMA-DPH. On the contrary, quenching decreases. Again, this result can be explained by a location of TMA-DPH away from the headgroup, anchored with its charge in the phosphate region.

The motional characteristics of TMA-DPH in a biological membrane can be expected to reflect both bulk lipid properties and the presence of proteins [320]. To evaluate both factors, the fluorescence properties of TMA-DPH in sonicated platelets and vesicles from total platelet lipids were investigated (Fig. 5, tables I and II). In platelet lipid vesicles $\langle\tau\rangle$ and r_s of TMA-DPH were 5.00 ± 0.06 ns and 0.226 ± 0.003 (mean $\pm$ s.d., $n=7$). These values are much lower than those from the outer monolayer of the platelet plasma membrane, again indicative for a fluidity gradient over the plasma membrane. In sonicated platelets $\langle\tau\rangle$ and r_s of TMA-DPH amounted to 5.39 ± 0.03 ns and 0.241 ± 0.002 (mean $\pm$ s.d., $n=3$). The higher order in sonicated platelets with respect to platelet lipid vesicles can be explained by the presence of membrane proteins, which are known to increase the lipid order in bilayers [105, 321, 620].

To investigate the relative importance of individual membrane lipid components in determining lipid order as sensed by TMA-DPH, a systematic survey of the major lipid species of

the platelet plasma membrane was performed. From the data presented in Fig. 5 and Tables I and II, it can be seen that fluorescence lifetime and anisotropy of TMA-DPH in PC vesicles have much smaller values than those in all platelet systems. Inclusion of 30 mole% PE, PS, or SphM shows that these lipids are not major determinants of fluorescence lifetime and anisotropy of TMA-DPH in lipid bilayers, and that SphM is more effective than PE or PS. Inclusion of 58 mole% cholesterol (C/P ratio 1.4) clearly shows that cholesterol is by far the major determinant of fluorescence lifetime and anisotropy of TMA-DPH in lipid bilayers, even when the higher molar level of cholesterol (58%) relative to PE, SphM, and PS (30%) is taken into account. The importance of cholesterol for the fluorescence properties of TMA-DPH is also demonstrated by platelet lipid vesicles, which contained 35 mole% cholesterol. A similar important role of cholesterol for the fluorescence properties of TMA-DPH in lipid bilayers is reported by Straume & Litman [618] and by Deinum et al. [155]. It should be mentioned that the unsaturation of phospholipid acyl chains also affects lipid order as sensed by TMA-DPH [155, 617]: more unsaturation results in decreased lipid order. Quantitatively, the effect of acyl chains on fluorescence lifetime and lipid order is relatively small, but may be responsible for differences observed between PC, PS, and PE.

Special attention is required by the dynamic component of fluidity, expressed as the rotational diffusion coefficient ($D_{\perp}$). This parameter changed during penetration of TMA-

Table IV. Differential influence of major membrane components on fluorescence lifetime and anisotropy of TMA-DPH.

membrane component	$\langle\tau\rangle$	r_s	S	$D_{\perp}$
protein	-	+	+	-
sphingomyelin	+	-	+	-/+
acyl-chain unsaturation	-	-	-	+
cholesterol	++	++	++	++

This table is a qualitative representation of Fig. 5, designating the relative contribution of major membrane components to fluorescence lifetime and motional properties of TMA-DPH. The relative contribution can either be of minor importance (-), of significant importance (+), or of major importance (++) to the parameter in question.

DPH into unstimulated platelets in a way different from the other parameters (Fig. 4). $D_{\perp}$ very rapidly increases to a high plateau-like level, while the other parameters change smoothly during penetration of TMA-DPH into platelets. In addition, even the highest rate of motion, observed with egg-PC alone, is still lower than $D_{\perp}$ in platelets after more than one hour labeling with TMA-DPH (60 vs 80 MHz). Apparently, $D_{\perp}$ senses a specific aspect of intracellular platelet membranes which is not revealed by the other parameters. Neither SphM nor cholesterol can be responsible for these fast movements, considering their enrichment in the outer monolayer. Intracellular proteins also have to be excluded, since proteins decrease

motional rates due to their own much slower motion. Lowering the extent of acyl chain unsaturation is accompanied by slower motion of TMA-DPH [617]. Thus, the high $D_{\perp}$ values observed when TMA-DPH enters the platelet interior, can be explained by the specific enrichment of arachidonic acid in the inner monolayer of the platelet plasma membrane [494].

Fig. 5. Fluorescence lifetime and motional properties of TMA-DPH in various membrane systems. Presented are the average fluorescence lifetime, $\langle \tau \rangle$, the steady-state anisotropy, r_s , the structural order parameter, S , and the rotational diffusion constant, $D_{\perp}$. Membrane systems used are: (A) unstimulated platelets immediately after labeling, (B) sonicated platelets, (C) platelet lipid vesicles, (D) PC vesicles, (E) PC/PE (molar ratio 7/3) vesicles, (F) PC/PS (7/3) vesicles, (G) PC/SphM (7/3) vesicles, (H) PC/cholesterol vesicles, C/P ratio 1.4. Platelet preparations were equivalent to $2 \cdot 10^7$ platelets per ml. Vesicle preparations contained 25 to 50 μ M phospholipid per ml. Experimental details are described in the methods. Data shown are the mean $\pm$ s.d. of at least 3 separate measurements.

Further characterization of the effect of cholesterol on the behavior of TMA-DPH in membranes

Since it was found that cholesterol is the major determinant of both fluorescence lifetime and anisotropy of TMA-DPH in membranes, the effects of cholesterol were considered in more detail by investigation of its dose-response relationship. It was found that by increasing the cholesterol content in PC vesicles from zero to a C/P ratio of 1.8, $\langle \tau \rangle$ increased from 2.63 ± 0.03 to 7.17 ± 0.04 ns (mean $\pm$ s.d., $n=3$), while S increased from 0.600 ± 0.004 to 0.900 ± 0.003 (mean $\pm$ s.d., $n=3$). Unlike $\langle \tau \rangle$, which increases almost linearly with cholesterol content, S clearly levels off at C/P ratios higher than 1. The relation between cholesterol content and S is in line with the observation that the condensing effect of cholesterol [159], and its effect on microviscosity [131] and steady-state fluorescence polarization [62] level off at high C/P ratios. The dependence of r_s on cholesterol content in PC

vesicles obviously combines the behavior of S and $D_{\perp}$, as could be expected from its nature.

The relationship between cholesterol content and the rotational dynamics of TMA-DPH was found to be biphasic: $D_{\perp}$ increases at low cholesterol concentrations from about 60 to 70 MHz, followed by a decrease down to about 10 MHz at a C/P ratio of 1.8. This biphasic behavior has not been observed before, and may be related to the specific location of TMA-DPH in the lipid bilayer. In general an inverse relationship between S and $D_{\perp}$ is observed, explaining the decreasing part of the $D_{\perp}$ curve. The increase in $D_{\perp}$ of TMA-DPH with cholesterol at lower C/P ratios is in agreement with findings from Straume & Litman [618], who used various PC vesicles with C/P ratios up to 0.4. The peak of $D_{\perp}$ around 20 mole% cholesterol may be explained by its concentration-dependent organization in lipid bilayers of PC, which changes significantly around 20 mole% [for review see: 325, 579]: below about 20 mole% cholesterol occurs in cholesterol-rich domains, while above 20 mole% cholesterol is homogeneously distributed, and at even higher cholesterol

Fig. 6. Fluorescence lifetime and motional properties of TMA-DPH in PC vesicles with various amounts of cholesterol. Presented are the average fluorescence lifetime, $\langle \tau \rangle$, the steady-state anisotropy, r_s , the structural order parameter, S , and the rotational diffusion constant, $D_{\perp}$. Data shown are the mean of two separate measurements. Standard deviations fall within symbols. Dotted lines represent the average values of TMA-DPH in unstimulated platelets immediately after labeling. Experimental details are described in the methods.

rol levels pure cholesterol domains may separate out.

DISCUSSION

Application of TMA-DPH as a reporter of structural membrane properties, and time-resolved fluorescence spectroscopy to monitor and analyze both fluorescence lifetime and static as well as dynamic components of fluidity, appeared to present a versatile combination allowing extrapolation of fundamental properties of membrane fluidity to biological membranes, and revealing detailed information about the organization of platelet membranes. Following the fate of TMA-DPH after addition to unstimulated platelets, a gradual penetration into the platelet interior could be demonstrated both by fluorescence intensity measurements, and by making use of the sensitivity of time-resolved fluorescence. Hyperbolic fitting of both the increase in non-dilutable F (Fig. 1B) and the increase in membrane-bound fraction of TMA-DPH (Fig. 3), shows that after approximately 6 hours all platelet membranes will be labeled. Halfmaximal penetration of TMA-DPH into platelets is obtained after 2 hours.

The gradual penetration of TMA-DPH is accompanied by progressive changes in both fluorescence lifetime and anisotropy of TMA-DPH, indicating that the intracellular platelet membranes are much more fluid than the outer monolayer of the platelet plasma membrane. In general, the observed difference in fluidity can be due to quantitative changes in one or more of the following membrane components: (1) cholesterol [62, 155, 313, 320, 618], (2) sphingomyelin [25, 62], (3) acyl chain unsaturation [62, 155, 320, 617, 621], and (4) membrane proteins [105, 320, 321].

Cholesterol is not evenly distributed over the various platelet membranes. Membranes from the dense tubular system contain less cholesterol than the plasma membrane [186, 195, 243, 358, 418]: C/P ratio about 0.4 vs. 0.7, respectively. Secretory granules contain even less cholesterol [243, 556]: C/P ratio between 0.20 and 0.25. In view of the dramatic effects of cholesterol on membrane fluidity (Figs. 5 and 6), it is clear that a decrease in cholesterol content in intracellular membranes will be responsible for

a significant part of the observed fluidity gradient.

Sphingomyelin is also not homogeneously distributed throughout the platelet. The platelet plasma membrane is enriched in sphingomyelin [195, 358, 556]. Again, such a distribution of sphingomyelin would favor the observed increase in fluidity of intracellular membranes, in view of its effects on membrane fluidity (Fig. 5). The contribution of sphingomyelin to the fluidity gradient is enhanced by its extreme asymmetric distribution over the plasma membrane. More than 90% of sphingomyelin in the platelet plasma membrane is located in the outer monolayer [98, 494].

Acyl chain unsaturation will further augment the fluidity gradient. About 60% of all esterified arachidonic acid in platelets is present in the plasma membrane, the majority of which residing in the cytosolic leaflet [494]. The contribution of acyl chain unsaturation to the observed fluidity gradient in platelets is further demonstrated by the observation that polyunsaturated fatty acids provide about 30% of the acyl chains in the sn-2 position of phospholipids in the outer monolayer of the platelet plasma membrane [494], while total platelet phospholipids contain about 50% polyunsaturated fatty acids in the sn-2 position [396]. Direct evidence for the involvement of acyl chain unsaturation in the fluidity gradient can be obtained from the changes in rotational diffusion constant ($D_{\perp}$) during gradual penetration of TMA-DPH into platelets. During penetration of TMA-DPH into unstimulated platelets (Fig. 4), $D_{\perp}$ increases to levels higher than obtained with cholesterol-free PC vesicles (Fig. 5). It was argued, in agreement with other observations [617], that an increase in acyl chain unsaturation can cause these high rotational rates. In this way, earlier observations about transbilayer asymmetry of acyl chain unsaturation [494] are confirmed. Another possibility which should be considered, is the interaction between cytosolic proteins with intracellular membranes (vide infra).

Proteins decrease membrane fluidity, due to their relative slow motional characteristics in comparison with lipids [105, 321]. This is evident from the difference between sonicated platelets and vesicles from platelet lipids (Table II). Thus, proteins would counteract the observed fluidity gradient in platelets. Besides membrane proteins, cytosolic proteins could, in

principle, also influence the fluorescence properties of TMA-DPH. This is demonstrated by the binding of TMA-DPH to albumin [166], which is characterized by a very high anisotropy. A contribution of binding of TMA-DPH to cytosolic proteins, however, seems unlikely for the following reasons. During extensive penetration of TMA-DPH into platelets, the steady-state anisotropy approaches values obtained only in membranes free of cholesterol and proteins (Fig. 5), excluding an involvement of cytosolic proteins in the fluorescence properties of TMA-DPH, since proteins significantly increase lipid order [105, 321]. In addition, the increase in binding of TMA-DPH to platelets during its penetration is in reasonable agreement with the increase which would be expected from the ratio of phospholipids between outer monolayer and total platelets.

From the above discussion it is obvious that the observed increase in membrane fluidity during penetration of TMA-DPH into unstimulated platelets is in line with current findings about the compositional distribution of lipids throughout the platelet. Generally speaking, the platelet intracellular membranes must have a fluidity approaching that of egg-PC vesicles, while the outer monolayer displays a membrane fluidity only found with vesicles containing more than 50 mole% cholesterol (compare Figs. 4 and 5). Detailed conclusions about the relative contributions of the various intracellular membranes to the observed fluidity gradient can not be extracted from the averaged fluorescence lifetime and motional properties of TMA-DPH. Moreover, considering the rapid equilibrium between membrane and aqueous compartments of TMA-DPH, it is anticipated that intracellular TMA-DPH will diffuse rapidly throughout the platelet, providing only aspecific information about the fluidity of intracellular platelet membrane compartments.

Owing to the specific initial incorporation of TMA-DPH in the outer monolayer of the platelet plasma membrane, however, more detailed information about the membrane fluidity of the outer leaflet of the plasma membrane can be obtained. From the data presented in Fig. 5, several conclusions can be inferred designating the relative importance of the various membrane components in determining the fluorescence properties of TMA-DPH in membranes (Table IV). Comparison of sonicated platelets and platelet lipid vesicles shows

that the presence of membrane proteins is especially reflected by r_s and S , while $\langle \tau \rangle$ and $D_{\perp}$ are less influenced by proteins. Judged from S and r_s , it appears that proteins do not contribute much to the membrane fluidity of the outer monolayer. Of the lipid components, only cholesterol has major effects on membrane fluidity, as judged by all parameters. Sphingomyelin is less effective, especially when r_s is evaluated. Acyl chain unsaturation is also of minor importance relative to cholesterol, except for its effect on $D_{\perp}$. Thus it can be concluded that cholesterol is the most important determinant of the fluidity of the outer monolayer of the platelet plasma membrane, suggesting that the fluidity gradient over the plasma membrane is mainly due to an asymmetric transbilayer distribution of cholesterol.

Quantitative conclusions about the cholesterol content of the outer leaflet of the platelet plasma membrane may be drawn from the cholesterol dose-response relationships. The fluorescence lifetime of TMA-DPH seems most appropriate to relate to cholesterol content, since it is hardly sensitive to proteins and acyl-

Fig. 7 Estimation of the C/P ratio in the outer monolayer of the platelet plasma membrane from the fluorescence lifetime of TMA-DPH in cholesterol containing vesicles. The solid line represents the cholesterol-dependence of the average fluorescence lifetime of TMA-DPH in vesicles containing the following mixture of phospholipids: SphM/PC/PE (6/3/1, molar ratio). All phospholipids were from egg-yolk (Sigma, St. Louis MO, USA). The upper dotted line represents the outer monolayer of the platelet plasma membrane as sensed by TMA-DPH. The lower dotted line is derived from the upper one by subtraction of the difference between sonicated platelets and platelet lipid, taken to represent the maximal contribution of proteins. Data were taken from a representative experiment. Experimental details are described in the methods.

chain unsaturation (Table IV). Egg-PC vesicles, however, do not qualify as representative for the outer monolayer of the platelet plasma membrane. Therefore, the cholesterol dependence of the lifetime of TMA-DPH was determined in vesicles containing SphM, PC and PE in a molar ratio of 6, 3 and 1, respectively, resembling the outer leaflet of the plasma membrane [98, 494]. It can be seen in Fig. 7 that the lifetime of TMA-DPH in the outer leaflet would reflect a C/P ratio of 1.15, if the effect of proteins is not taken into account. If the difference between sonicated platelets and platelet lipid vesicles is taken to represent the effect of proteins on the fluorescence lifetime of TMA-DPH, then a C/P ratio of 0.75 is estimated for the outer leaflet. It can be argued, however, that the correction for the effect of proteins is an overestimation. Most of the difference in fluorescence lifetime of TMA-DPH between sonicated platelets and platelet lipid vesicles is due to intracellular proteins, as is suggested by the effect of Ca^{2+} -dependent proteolysis of cytosolic proteins prior to sonication (see next chapter). Thus, the best estimate for the cholesterol content of the outer monolayer of the platelet plasma membrane would be a C/P ratio of about 1.

An independent estimation of the cholesterol content of the outer monolayer of the platelet plasma membrane can be obtained from the work of Shattil and Cooper [593]. These authors manipulated the cholesterol content of platelets by incubation with cholesterol-rich vesicles. They found that only vesicles with a C/P ratio higher than 1.0 were able to enrich platelets in cholesterol. Assuming a cholesterol exchange process between the outer monolayer of vesicles and platelets [567, 715], this finding suggests a C/P ratio of 1.0 for the outer leaflet of the platelet plasma membrane.

Another independent estimation of the cholesterol content in the platelet outer monolayer can be derived from the work of Donner and Stoltz [166], who used TMA-DPH to investigate platelets and erythrocytes. These authors found that TMA-DPH displays the same steady-state anisotropy in platelets and erythrocytes, immediately after labeling. Considering that the phospholipid composition of the outer monolayer of erythrocytes and platelets are similar [731], and that cholesterol is the major determinant of membrane fluidity in the erythrocyte [105], the similarity in anisotropy of

TMA-DPH is taken to indicate that the outer monolayer of both erythrocyte and platelets contain similar amounts of cholesterol. The C/P ratio of erythrocyte membranes is 0.85 to 0.90 [102, 105, 365]. In addition, cholesterol is asymmetrically distributed over the plasma membrane: approximately two-third is located in the outer monolayer [201, 261], equivalent to a C/P ratio of 1.1 to 1.2. Thus, comparison of platelets and erythrocytes suggests a C/P ratio for the platelet outer monolayer of at least 1.0.

Having estimated the cholesterol content of the outer leaflet of the platelet plasma membrane, the cholesterol content of the inner leaflet can be calculated from the C/P ratio of the whole plasma membrane. Values for the C/P ratio of the whole platelet plasma membrane vary from 0.6 [186] to 0.66 [243] and 0.74 [358, 418]. Taking a C/P ratio of 1.0 for the outer monolayer, as argued above, and a C/P ratio of 0.7 for the whole plasma membrane, it can be calculated that the C/P ratio of the inner monolayer would be about 0.4. This would constitute a considerable transbilayer asymmetry of cholesterol. It should be mentioned here that the suggested asymmetrical distribution of cholesterol is indirectly measured, being derived from the motional properties of TMA-DPH. Clearly, a direct determination, making use for instance of the fluorescent sterol dehydroergosterol [579], would be desirable, and should be employed to confirm the observed cholesterol asymmetry.

Transbilayer asymmetry of membrane fluidity has been observed in erythrocytes [163, 428, 587, 634, 703], in sarcoplasmic reticulum [429], and in fibroblasts [237, 318, 578, 707]. In comparison to the erythrocyte, the fluidity gradient over the platelet plasma membrane seems to be more extreme. This is suggested from the results of Donner and Stoltz [166], who observed that the time-dependent decrease in r_s of TMA-DPH, indicative for a fluidity gradient, was greater in platelets than in erythrocytes. An asymmetric transbilayer distribution of cholesterol is found to be associated with the observed transmembrane fluidity gradient in erythrocytes [184, 201, 261], and fibroblasts [261, 318]. In addition, transbilayer asymmetry of cholesterol is reported for Mycoplasma cells [59], and myelin [92]. Thus, the existence of a transmembrane fluidity gradient, coupled to an asymmetric transbilayer distribution of cholesterol, is not a rare phenomenon

and may turn out to be a general feature of the lipid organization in biological membranes.

The existence of a transmembrane cholesterol gradient raises several questions concerning its regulation and maintenance. The rate of transbilayer movement (flip rate) of cholesterol is very rapid, with a half-time of seconds [579, 715]. These high flip rates are not necessarily at odds with a stable transmembrane gradient of cholesterol, since additional factors may contribute to the maintenance of the gradient. Such factors could determine the equilibrium distribution of cholesterol, independent of the dynamics of cholesterol motion, by increasing the affinity for cholesterol of one monolayer. In view of the preferential affinity of cholesterol for sphingomyelin [158], the extreme asymmetric transbilayer distribution of sphingomyelin in the platelet plasma membrane [98, 494] could explain an enrichment of cholesterol in the outer monolayer. Membrane proteins provide another candidate for binding cholesterol. Significant binding of cholesterol to membrane proteins has been demonstrated in erythrocytes [586, 716], in brush border plasma membranes [63], and in model systems [17, 598]. Whether membrane proteins can bind cholesterol preferentially in one monolayer of a membrane is not known, leaving their role in maintenance of cholesterol transbilayer asymmetry uncertain.

The occurrence of a fluidity gradient over the platelet plasma membrane may contribute significantly to the functional asymmetry of the plasma membrane, which is outlined in the introduction. At the cytosolic side of the plasma membrane, the high fluidity will favor protein-lipid interactions [184], important for the action of phospholipases [157] and the binding of peripheral proteins, like cytoskeletal proteins [256, 458]. Interaction of cytoskeletal proteins with the cytosolic leaflet of the plasma membrane is likely to involve binding of amphipathic helices to the lipid bilayer surface [176,

665]. It is conceivable that shallow penetration of amphipathic helices into the lipid bilayer leads to spacing of headgroups resulting in a lower lipid order within the lipid bilayer. Decreased lipid order is accompanied by increased rates of motion [320, 620]. It is emphasized that $D_{\perp}$ increases rapidly during penetration of TMA-DPH into platelets to levels which are much higher than those found for cholesterol-free membranes. Thus, it is suggested that interaction of cytoskeletal proteins with intracellular membranes is a common phenomenon in platelets. Such interactions between cytoskeletal proteins and the lipids of the inner leaflet of the plasma membrane have been implicated in the maintenance of phospholipid transbilayer asymmetry [54, 733]. Thus, the high fluidity of the inner leaflet of the plasma membrane may be linked to the regulation of transbilayer lipid asymmetry.

The high viscosity of the outer monolayer clearly suits its function as a permeability barrier, since high levels of cholesterol result in low permeability of the lipid bilayer towards non-electrolytes [159]. In addition, incorporation of cholesterol decreases the flip rate of phospholipids [40, 424]. Thus, the high viscosity of the outer leaflet of the plasma membrane may contribute to the maintenance of phospholipid transbilayer asymmetry.

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CHAPTER 6

Rapid transbilayer movement in the platelet plasma membrane during cytoskeletal degradation by calpain

ABSTRACT

The structural integrity of the human platelet plasma membrane during stimulation of rapid transbilayer movement of phospholipids was investigated employing the fluorescent membrane probe TMA-DPH (trimethylammonium-diphenylhexatriene) as a reporter of both membrane structural properties and rates of aspecific transbilayer movement. Fluorescence intensity as well as time-resolved fluorescence were applied to describe the fluorescence properties and location of TMA-DPH. In unstimulated platelets transbilayer movement was relatively slow, with a halftime of 60 to 90 min. Stimulation of platelets by thrombin resulted in an increase of membrane-bound TMA-DPH, explained by the release reaction, and an increase of lipid order in the outer monolayer of the plasma membrane, but did not change transbilayer movement of TMA-DPH, and did not affect the presence of the fluidity gradient over the plasma membrane. Stimulation of platelets by ionomycin gave rise to rapid and massive transbilayer movement of TMA-DPH (halftime 10 to 20 sec), accompanied by a loss of the transmembrane fluidity gradient and an increased fluidity of the outer monolayer of the plasma membrane. The high rate of transbilayer movement during stimulation by ionomycin suggests the presence of local defects in lipid bilayer structure (flipsites). Further examination of flipsites during platelet stimulation by ionomycin showed that these sites were only temporarily present, and were Ca^{2+} -dependent with a halfmaximal activity around 20 μM free Ca^{2+} . Agonist-, time-, and Ca^{2+} -dependence of flipsites are similar to those of cytoskeletal degradation by calpain [Verhallen et.al. (1987), *Biochim.Biophys.Acta*, 903: 206], suggesting that cytoskeletal reorganization is involved in the generation of flipsites. This suggestion is further supported by the Ca^{2+} -dependent generation of flipsites in fluoride-treated platelets.

INTRODUCTION

The plasma membrane of the blood platelet is an interesting object to study the regulation of transbilayer lipid asymmetry. The lipids are unequally distributed among the two leaflets of the plasma membrane: phosphatidylethanolamine (PE) and phosphatidylserine (PS) are respectively enriched and almost totally located in the cytosolic leaflet, while the outer monolayer of the platelet plasma membrane is enriched in phosphatidylcholine (PC) and cholesterol, and contains almost all sphingomyelin (SphM) [98, 494, 569, previous chapter]. The phospholipid transbilayer asymmetry resembles that of the erythrocyte [666, 736]. In contrast to the erythrocyte, however, transbilayer asymmetry can be dramatically changed upon cell activation [53]. Depending on the stimulus, phospholipid transbilayer asymmetry of the plasma membrane is more or less lost during platelet activation [50]. As a result of this randomization of phospholipids, PS becomes exposed at the outer surface of the plasma membrane, where it accelerates blood clotting [543a]. The activation-dependent loss of phospholipid transbilayer asymmetry is a rapid phenomenon: when platelets are activated by a calcium ionophore, phospholipid asymmetry is completely lost within one minute [664].

In general, two different mechanisms can account for alterations in transbilayer lipid asymmetry: translocation mediated by an ATP-dependent transport protein (translocase) [128, 144, 406, 603, 643, 721, 722], and transbilayer movement (flipflop) along local disturbances of the lipid bilayer (flipsites) [39, 154].

The existence of a translocase in platelets has been demonstrated [623]. Although the translocase is normally observed by the unidirectional transport of aminophospholipids towards the cell interior, the possibility of outward transport has been suggested recently [702]. However, involvement of a translocase in the activation-dependent loss of transbilayer asymmetry of the platelet plasma membrane seems, *a priori*, unlikely for the following reasons: (1) the translocase specifically transports amino-phospholipids, while rapid flipflop in activated platelets also concerns SphM and PC [50]; (2) stimulation of rapid flipflop in platelets can be observed only at relatively high cytosolic

Ca^{2+} levels (higher than $1 \mu\text{M}$) [664], at which translocase activity is inhibited in erythrocytes [721]; (3) the inward fliprate in activated platelets ($t_{1/2}$ less than 1 min, [664]) is more rapid than outward transport rates of the translocase ($t_{1/2}$ for PS about 7 min at 37°C , [623]).

Transbilayer movement of phospholipids in model lipid systems is a slow process [476, 545], obstructed by a high activation energy to pass the charged headgroup through the hydrophobic core of the lipid bilayer. Flipflop can be accelerated by introducing different physical properties between outer and inner monolayer, by insertion of bilayer spanning proteins, and by triggering the formation of non-bilayer structures in the lipid bilayer [141, 154]. In erythrocytes, increased transbilayer movement is observed after reorganization of the interaction between cytoskeleton and plasma membrane [15, 39, 95, 103, 194, 217, 218, 423, 477, 526, 703]. Previously, we have demonstrated that rapid flipflop during platelet activation is dependent on the degradation of cytoskeletal proteins by calpain [124, 663, 664]. Thus, transbilayer movement in activated platelets could very well be due to the generation of flipsites during calpain induced reorganization of the interaction between cytoskeletal proteins and plasma membrane components.

The occurrence of flipsites in the plasma membrane during platelet activation was investigated employing the fluorescent membrane probe TMA-DPH. This probe has been reported to incorporate quickly in the outer monolayer of the plasma membrane [345, 348]. Due to a partition equilibrium [347], most of TMA-DPH will remain in solution, where it is hardly fluorescent [519]. Transbilayer movement of TMA-DPH [137] will result in labeling of intracellular compartments accompanied by more uptake of probe from solution which results in an increase in fluorescence intensity [347, 348]. Thus, the rationale was that loss of transbilayer asymmetry in platelets, which involves both inward and outward movement of phospholipids [50], would be accompanied by an increase in fluorescence intensity due to transport of TMA-DPH to intracellular membrane compartments.

MATERIALS AND METHODS

Platelet isolation

Human platelets were isolated, as outlined previously [51], by differential centrifugation from fresh blood drawn from healthy volunteers who received no medication. Blood was anticoagulated with acid-citrate-dextrose. Platelets were washed in an isotonic buffer at pH 6.6, containing 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM glucose (Hepes buffer) in the presence of 0.05% fatty acid-free human serum albumin. They were finally resuspended at a concentration of 2.10^8 per ml in Hepes buffer, pH 7.4, without albumin. Platelet concentration was inferred from their absorption at 405 nm using a calibration curve determined with a Coulter Counter. Platelet lysis was determined by assaying lactate dehydrogenase (EC 1.1.1.27) in a 12000g supernatant according to Wroblewski and La Due [710].

Total platelet membranes were prepared by sonication in the presence of 1 mM EGTA for 5 min, with 10 sec intervals, in a direct-probe sonicator (MSE) at an amplitude of 12 μ m.

Platelet lipid vesicles

Total platelet lipids were extracted according to Bligh and Dyer [61], from platelets pooled from 8 donors. Lipids were stored in chloroform/methanol (1/1, by vol.) at -20°C under N_2 . Aliquots of lipid solutions were evaporated to dryness under a stream of N_2 . Dry lipids were hydrated by addition of Hepes buffer (pH 7.4, without albumin), which was degassed with N_2 . Vesicles were prepared by sonication for 5 min at 40°C , with an amplitude of 14 μ m. After sonication, vesicles were centrifuged at 14,000g for 5 min to remove non-vesicular structures. The final phospholipid concentration was determined according to Böttcher et al. [66], and varied between 2 and 5 mM. Cholesterol concentration was determined enzymatically by the cholesterol oxidase assay, using a commercial kit (Sigma, St. Louis MO, USA).

Platelet activation

Washed human platelets were diluted in quartz cuvettes at 37°C in Hepes buffer, pH 7.4, containing 3 mM Ca^{2+} . At a concentration of 2.10^7 per ml, platelets were activated either by 2 nM thrombin (isolated and purified according to Rosing et al. [544]) or 0.5 μ M ionomycin (Calbiochem, USA) under moderate stirring (250 rev/min). In dilution experiments, platelets were activated at a concentration of 2.10^8 per ml by 4 nM thrombin or 1 μ M ionomycin in plastic tubes under otherwise similar conditions.

Ca^{2+} titration of intact platelets was carried out as described previously [664]. Extracellular free Ca^{2+} concentration was manipulated by a 30 mM HEDTA/ Ca^{2+} mixtures in Hepes buffer without magnesium. Intracellular Ca^{2+} was equilibrated with extracellular Ca^{2+} by addition of 0.5 μ M ionomycin to 2.10^7 platelets per ml in quartz cuvettes at 37°C under moderate

stirring. All Ca^{2+} concentrations were checked with a Ca^{2+} -selective electrode (Radiometer, Copenhagen, Sweden; type F2110Cc).

Ca^{2+} -dependent activation of fluoride-treated platelets was performed as outlined before [663]. Briefly, platelets were suspended to a concentration of 2.10^8 per ml in Hepes buffer, pH 7.4, containing 1 mM EGTA, and incubated with 10 mM sodiumfluoride (Merck, Darmstadt, FRG) without stirring at 37°C . At various time intervals, a subsample was diluted 10 times in Hepes buffer, pH 7.4, and 5 mM Ca^{2+} was added.

Fluorescence measurements

TMA-DPH (Molecular Probes Inc., Eugene OR, USA) was dissolved in N,N -dimethylformamide at a concentration of 5 mM and stored at -20°C in the dark. Washed human platelets were diluted into a 3 ml quartz cuvette with Hepes buffer pH 7.4 to a concentration of 2.10^7 per ml and labeled at 37°C with 1 μ M TMA-DPH under moderate stirring, using a double dilution procedure described in the previous chapter. Sonicated vesicles were employed at a concentration between 25 and 50 μ M, under the same conditions as platelets. All agents used during fluorescence measurements were tested for their effect on the fluorescence properties of TMA-DPH, and were found to be without any effect unless explicitly mentioned.

Fluorescence intensities (F) were recorded at 37°C under moderate stirring with an SLM Aminco SPF-500C fluorimeter equipped with a light-tight injection port and a stirring device (Hellma). Excitation and emission wavelengths were set at 365 and 450 nm with 5 and 20 nm slits, respectively. F from light-scatter or background fluorescence varied between 1 and 2%, and was neglected.

Phase fluorimetry was performed on a SLM-4800 fluorimeter according to Prendergast et al. [519], using a POPOP reference solution in order to remove any color-dependent response of the detector [361]. Lifetimes from phase shifts (τ_p) were determined at a frequency of 30 MHz, by a cross-correlation method using the computer program supplied by the manufacturer. Excitation was accomplished using a monochromator set at 360 nm (0.5 nm bandpass). Emission was passed through two KV408 cut-off filters (Schott) before detection. Background fluorescence and light scatter amounted to less than 1% of total intensity and were neglected. All measurements were performed at 37°C without stirring.

Fluorescence decay and fluorescence anisotropy decay were determined with a laser-pulsed single photon counting fluorimeter, described in detail elsewhere [284, 285, 286, 671]. TMA-DPH was excited at 310 nm by a frequency-doubled, synchronously pumped rhodamine 6G dye laser. Emission was monitored above 450 nm through a K45 band-pass filter (Balzers). All measurements were performed at 37°C without stirring.

Analysis of time-resolved data

Data were analyzed as described in the previous chapter. The fluorescence decay of TMA-DPH was routinely analyzed by a biexponential function. In addition, a tetra-exponential function, fitting from lower channel numbers, was used to include the lifetime of TMA-DPH in solution.

The average fluorescence lifetime, $\langle \tau \rangle$, was calculated from the individual components from:

$$\langle \tau \rangle = \Sigma a_i \tau_i^2 / \Sigma a_i \tau_i$$

The anisotropy decay function, $r(t)$, of TMA-DPH in membranes was interpreted within the framework of the "wobbling-in-cone" model introduced by Kinosita et.al. [320, 322]:

$$r(t) = r_\infty + (r_0 - r_\infty)e^{-(t/\phi)}$$

where r_0 is the fundamental anisotropy, r_∞ is the residual anisotropy at the end of the experiment (about 30 ns), and ϕ is the apparent relaxation time.

Membrane fluidity, as sensed by TMA-DPH, is expressed in a static and a dynamic component. The static component is expressed by the second-rank order parameter, S , and obtained from:

$$S = \sqrt{(r_\infty/r_0)}$$

The cone-angle, θ_c , describing the range of acyl-chain motion in the "wobbling-in-cone" model, is determined from:

$$S = 1/2 \cos \theta_c (1 + \cos \theta_c)$$

The dynamic component is expressed by the model-independent diffusion constant for rotation around an axis perpendicular to the probe symmetry axis, $D_\perp$, and is evaluated from:

$$D_\perp = (r_0 - r_\infty)/(6r_0\phi)$$

assuming that the anisotropy decays exponentially to r [9].

To accommodate the frequent use of steady-state anisotropy, r_s , to describe changes in fluidity, r_s was also derived using the Perrin-equation:

$$r_s = r_\infty + (r_0 - r_\infty)/(1 + \tau/\phi)$$

in which τ was substituted by the average fluorescence lifetime, $\langle \tau \rangle$, from the biexponential fit, which excludes any contribution of the ultrafast fluorescence of TMA-DPH in buffer. In addition, steady-state anisotropy can be expressed as the degree of polarization, P , related to r_s :

$$P = 3r_s/(2 + r_s)$$

S , $D_\perp$, and r_s were calculated with an average r_0 obtained from 96 experiments, as described in the previous chapter.

RESULTS

Transbilayer movement of TMA-DPH during platelet activation.

Addition of TMA-DPH to unstimulated platelets results in an immediate rise in fluorescence intensity (F) (Fig. 1A). This increase in F is due to membrane-bound TMA-DPH, which has a much higher average fluorescence lifetime, $\langle \tau \rangle$, than TMA-DPH in aqueous solution [previous chapter]. After the initial rise of F , we consistently observed a moderate decline, leveling off to a stable signal within a few minutes. This drop in F during illumination is also observed when TMA-DPH is added to Hepes buffer only, which suggests that it is a property of TMA-DPH fluorescence itself. Rapid bleaching of TMA-DPH upon illumination has been observed with fluorescence microscopy [80, 348], and suggested to be due to photoisomerization during the excited state [173]. Therefore, we suggest that photoisomerization causes the decrease in F , and that this decline reaches a steady-state equilibrium under moderate stirring.

A few minutes after addition of TMA-DPH to platelets, more than 96% of total TMA-DPH is still in solution (Table III, a_1). The membrane-bound TMA-DPH is most likely located in the outer leaflet of the plasma membrane, since upon sonication the amount of TMA-DPH in solution decreases further to about 82% (Table III), suggesting that most of the platelet membranes are not labeled immediately after addition of TMA-DPH to intact platelets. The increase in membrane-bound fraction of TMA-DPH is accompanied by a twofold increase in F (Fig. 1A). It is emphasized that F cannot be directly interpreted as number of fluorescent molecules, since F is also dependent on $\langle \tau \rangle$, which decreases upon platelet sonication (Table II), indicating that the increase in F is an underestimation of the increase in membrane-bound TMA-DPH. The suggestion that TMA-DPH is initially located solely in the outer monolayer of platelets, is supported by observations with fluorescence microscopy which show that TMA-DPH initially labels only the plasma membrane [80, 348], which may be interpreted as the outer monolayer considering the rapid

equilibrium of TMA-DPH between membranes and aqueous solution. Thus, under the conditions employed, only the outer leaflet of the platelet plasma membrane is labeled with TMA-DPH, and any increase in *F* would indicate unlabeled membrane becoming available for binding by TMA-DPH.

Fig. 1. Changes in membrane-bound pools of TMA-DPH during platelet activation.

(A): Washed human platelets were labeled with TMA-DPH in Hepes buffer in the presence of Ca^{2+} (thin arrow). After stabilization of the fluorescence signal, platelets were left unstimulated (1), stimulated by 2 nM thrombin (2) or by 0.5 μM ionomycin (3), as indicated by the bold arrow. At the end of the measurement each platelet preparation was sonicated (dashed line), and *F* was measured again using the same instrumental settings. (B,C): Subsamples from the very same platelet preparations used in panel A were 100 times diluted (arrow) in Hepes buffer without TMA-DPH, before (B) and after (C) sonication. The instrumental signal magnification was hundredfold increased with respect to the settings used for panel A. The dashed horizontal lines indicate *F* of each platelet preparation before dilution, and represent 1% of *F* in panel A, just before (B) or after (C) sonication. Trace numbers are the same as in panel A. Recordings shown are representative for six experiments. All manipulations were carried out at 37°C under moderate stirring. For further details is referred to the methods.

When platelets, which are labeled with TMA-DPH, are activated by 2 nM thrombin, *F* increases about 45% (Fig. 1A). This has already been observed by Kubina et al. [345], who correlated this increase in *F* with serotonin release, suggesting increased incorporation of TMA-DPH in the platelet plasma membrane due to exocytosis. Platelet lysis was 1.5%, which is too small to account for the increase in *F*. Fluorescence decay analysis of TMA-DPH, added after platelet activation by thrombin, shows that the bound fraction of TMA-DPH increases from 3.6 to 5.6% (Table III). This increase is very well comparable to the increase in phospholipid hydrolysis in the outer monolayer of the platelet plasma membrane by exogenous sphingomyelinase and phospholipase A₂ upon activation by thrombin (from 21 to 33% of total phospholipids) [50]. The similar increase in membrane-bound TMA-DPH and hydrolysable phospholipids in the outer leaflet of the plasma membrane upon platelet stimulation by thrombin supports the notion that the increase in *F* is solely due to an increased amount of plasma membrane lipids after fusion with granules. More conclusive evidence for the location of TMA-DPH in platelets after stimulation of platelets by thrombin can be obtained from dilution experiments, which are based on the rapid equilibrium distribution of TMA-DPH between membranes and aqueous solution was employed. It was found that within 20 sec after dilution of unstimulated platelets, *F* reached a new steady-state level (Fig. 1B). Thrombin-activated platelets reached a similar steady-state level of *F* after dilution as unstimulated platelets, suggesting that TMA-DPH does not cross the plasma membrane during stimulation by thrombin. To verify whether stimulation by thrombin changes the TMA-DPH binding properties of the platelet plasma membrane, both unstimulated and thrombin-stimulated platelets were sonicated before dilution. No difference in steady-state level after dilution of the sonicate could be observed (Fig. 1C), indicating that the increase in *F* of TMA-DPH during stimulation of platelets by thrombin is not due to increased uptake capacity of the plasma membrane. Thus, the increase in *F* upon activation of platelets with thrombin seems to be related only to exocytosis.

Activation of platelets by the 0.5 μM ionomycin resulted in an increase in *F* of TMA-DPH of about 125% (Fig. 1A). This increase in *F*

upon activation is much higher than with thrombin, and does not correlate with serotonin release [345]. Sonication of the platelets activated by ionomycin does hardly increase F (Fig. 1A), which indicates that all platelet membranes become labeled by TMA-DPH during activation by the Ca^{2+} ionophore. Platelet lysis was 2.6%. These findings strongly suggest that TMA-DPH labels all membranes within platelets during activation by ionomycin. Dilution of ionomycin-activated platelets reveals the presence of much larger non-dilutable pool of TMA-DPH compared to unstimulated or thrombin-activated platelets (Fig. 1B), which most likely results from penetration of TMA-DPH into platelets during stimulation by ionomycin. The increase in F and non-dilutable F during activation of platelets by ionomycin may, however, be due to increased uptake and possible irreversible binding of TMA-DPH in the ionomycin containing plasma membrane. This possibility can be excluded, however, since platelets sonicated after activation by ionomycin show a similar steady-state level of F after dilution as do unstimulated platelets (Fig. 1C). Thus, it is concluded that flipflop of TMA-DPH over the platelet plasma membrane is the cause for the high levels of F observed after stimulation by ionomycin.

Flipflop of TMA-DPH in ionomycin-activated platelets may be caused by membrane disturbances induced by the presence of ionomycin itself in the platelet plasma membrane. Ionomycin is applied at $0.5 \mu\text{M}$, and at the platelet concentration used the phospholipid concentration was found to amount to $8 \mu\text{M}$. Assuming that all ionomycin will dissolve into membranes, a phospholipid/ionomycin ratio of 16 clearly suggests effects of ionomycin on membrane order. The possibility that ionomycin itself affected lipid order was investigated using platelet lipid vesicles, to avoid complications due to cell activation. It was found that addition of ionomycin to a suspension of platelet lipid vesicles did increase lipid order (S) moderately (about 1%), irrespective of the presence of Ca^{2+} or EGTA (Table I). Increase in lipid order has been suggested to be a general effect of molecules that dissolve in membranes [287]. Thus, it can be concluded that ionomycin itself does not decrease lipid order, suggesting that the structural integrity of the membrane is not impaired, and that rapid transbilayer movement of TMA-DPH in iono-

mycin-activated platelets is caused by processes secondary to the addition of ionomycin.

Alterations of the transbilayer fluidity gradient during platelet activation.

Time-resolved fluorimetry revealed that, in addition to changes in F , platelet activation by thrombin resulted in an increased steady-state anisotropy of the plasma membrane: r_s increased from 0.260 ± 0.005 to 0.278 ± 0.005

Fig. 2. Penetration of TMA-DPH into resting and activated platelets. (A) Platelets were activated as described in the methods. $1 \mu\text{M}$ TMA-DPH was added to unstimulated platelets (●), or two minutes after activation by thrombin (▲) or ionomycin (■). Only during stimulation and just before measurements, platelets were stirred. The fraction of TMA-DPH bound to platelets was determined as $(1-\alpha_1)$ from the tetraexponential fluorescence decay analysis. (B) Flipflop of TMA-DPH in ionomycin-activated platelets. TMA-DPH was added (arrow) two min after ionomycin. Experimental details are described in the methods. Data shown are representative for at least 3 experiments.

(mean $\pm$ s.d., $n=5$ and $n=4$, resp.). This increase in r_s is in good agreement with the work of Kubina et.al [345], who reported an increase in r_s of TMA-DPH from 0.275 to 0.295 upon stimulation with thrombin. The increase in r_s upon activation by thrombin was not accompanied by any change in the average lifetime of TMA-DPH, which remained 6.8 ± 0.1 ns ($n=4$). It is noted that these changes in fluidity and lifetime are confined to the outer monolayer of the platelet plasma membrane, considering the absence of transbilayer movement.

In contrast to thrombin, platelet activation by ionomycin resulted in a decrease in steady-state anisotropy from 0.260 ± 0.005 to 0.221 ± 0.003 (mean $\pm$ s.d., $n=5$ and $n=7$ resp.). Again, this is in good agreement with an earlier reported values of 0.275 and 0.240, respective-

ly [345]. The increase in fluidity was accompanied by a decrease in average lifetime from 6.8 ± 0.1 to 5.1 ± 0.1 ns (mean $\pm$ s.d., $n=5$ and $n=7$, resp.), as determined by the biexponential fluorescence decay fit.

In view of findings presented in the previous chapter, which demonstrate the existence of a fluidity gradient over the plasma membrane of unstimulated platelets, it was of interest to find out what happens to this fluidity gradient during platelet activation. At first glance, it can be suggested from the steady-state anisotropies that the fluidity gradient is not much changed in thrombin-activated platelets, but must be reduced in ionomycin-activated platelets. Possible alterations of the fluidity gradient during platelet activation were investi-

Fig. 3. Changes in lifetime and motional properties of TMA-DPH accompanying its penetration into resting platelets (●) and platelets activated by thrombin (▲) or ionomycin (■). Presented are the average lifetime, $\langle \tau \rangle$, the steady-state anisotropy, r_s , the structural order parameter, S , and the rotational diffusion constant, $D_{\perp}$. Experimental conditions are the same as in Fig. 2. Data shown are representative for 3 experiments.

gated by following the fluorescence properties of TMA-DPH during penetration into platelets.

First of all, the rate of penetration of TMA-DPH into activated platelets was compared with penetration into unstimulated platelets. It can be seen in Fig. 2, that uptake of TMA-DPH by thrombin-activated platelets follows a similar time course as uptake by unstimulated platelets, suggesting uptake by slow transbilayer movement. Uptake of TMA-DPH by ionomycin-activated platelets was quite different: after addition of TMA-DPH, the bound fraction quickly increased to a maximum level within 15 to 20 min. This upper level is similar to that of sonicated platelets (Table III), and seems to represent completely labeled platelets. Thus, it is suggested that flipflop of TMA-DPH over the platelet plasma membrane is considerably increased in ionomycin-activated platelets relative to unstimulated platelets and thrombin-activated platelets. The changes in F after addition of TMA-DPH to ionomycin-activated platelets (Fig. 2B), suggest that the fliprate of TMA-DPH in ionomycin-activated platelets is characterized by a $t_{1/2}$ of about 10 minutes.

The effect of platelet activation on the fluidity gradient over the plasma membrane was examined by following the motional properties and the lifetime of TMA-DPH during penetration into activated platelets. It is shown in Fig. 3, that during prolonged incubation of thrombin-activated platelets, $\langle \tau \rangle$ decreased identically to unstimulated platelets. The motional properties of TMA-DPH in thrombin-activated platelets changed in a manner identical to unstimulated platelets, though the curve was shifted to higher order in thrombin-activated platelets, due to higher starting levels. The changes in motional properties of TMA-DPH during penetration into thrombin-stimulated platelets are characterized by a decrease in order (S) and an increase in rate of motion ($D_{\perp}$) on a time scale of several hours. Thus, stimulation by thrombin does not alter the fluidity gradient in platelets.

Prolonged incubation of ionomycin-activated platelets with TMA-DPH showed completely different results (Fig. 3). Lifetime as well as motional properties did not change much during incubation with TMA-DPH. $\langle \tau \rangle$ and $D_{\perp}$ slowly decreased from 5.3 to 5.2 ns and from 54 to 51 MHz, respectively. Lipid order (S) and steady-state anisotropy (r_s) increased from 0.79 to 0.81 and from 0.222 to 0.231, re-

spectively. It is emphasized that penetration of TMA-DPH in ionomycin-activated platelets is rapid: the final level is reached in about 20 min (Fig. 2B). During this time, the major part of the increase in S and r_s occurred (Fig. 3). Apparently, the fluidity gradient observed in unstimulated platelets is lost upon activation by ionomycin.

Temporary character of flipsites.

As already pointed out, after stimulation of platelets with ionomycin, transbilayer movement of TMA-DPH is enhanced with respect to unstimulated platelets: $t_{1/2}$ of flipflop decreases from about 1 hour (previous chapter) to 10 min (Fig. 2A). On the other hand, during stimulation by ionomycin, TMA-DPH becomes translocated into the platelet almost towards the maximum level within one minute (Fig. 1 and Table III). Thus, rapid flipflop of TMA-DPH during activation by ionomycin seems to be enabled only temporarily. This suggestion was further examined by comparing the fluorescence intensity of TMA-DPH, added before or after stimulation by ionomycin. Quantitative interpretation of differences in F after activation of platelets by either ionomycin or thrombin is allowed since the average lifetime is the same, both for addition of TMA-DPH after and before activation (Table II). It was found that when TMA-DPH was added one minute after ionomycin, F increased only about 40% above basal level instead of about 90% when TMA-DPH was added before ionomycin (Fig. 4A). Addition of TMA-DPH to platelets before or after thrombin did not make any difference (Fig. 4B).

The temporary character of rapid transbilayer movement of TMA-DPH during activation of platelets by ionomycin was further detailed by adding TMA-DPH at various time intervals after ionomycin. It is shown in Fig. 4C that during 30 sec after addition of ionomycin the increase in F gradually drops. Addition of TMA-DPH more than 30 sec after ionomycin did not result in lower levels of F reached one minute after addition of TMA-DPH (not shown). Thus, it is suggested that rapid flipflop, as sensed by TMA-DPH, is present in ionomycin-activated platelets only for about 30 sec.

Ca^{2+} -dependence of flipsites.

Rapid transbilayer movement of TMA-DPH over the platelet plasma membrane during activation by ionomycin, but not by thrombin, resembles the flipflop of phospholipids in its agonist-dependence [50, 124]. We have shown previously that flipflop of phospholipids in activated platelets is dependent on the action of calpain towards cytoskeletal proteins [124, 663, 664]. To investigate the involvement of calpain in the generation of flipsites during platelet ac-

tivation by ionomycin, we determined the Ca^{2+} -dependence of fast transbilayer movement of TMA-DPH, following a previously described procedure [664]. Intracellular free Ca^{2+} was manipulated by applying buffered free Ca^{2+} levels outside and employing ionomycin to equilibrate intra- and extracellular free Ca^{2+} levels. For comparison with the Ca^{2+} -dependence of platelet procoagulant activity and calpain [664], the development of F upon addition of ionomycin was monitored for 5 min.

Addition of ionomycin to platelets suspended in HEDTA/ Ca^{2+} buffers resulted in a biphasic increase in fluorescence intensity of TMA-DPH (Fig. 5A). The first increase was observed with all Ca^{2+} levels, and correlates with the release reaction, which was found to be elicited by ionomycin at any Ca^{2+} level. The second, and potentially much larger, increase in F was dependent on free Ca^{2+} levels; at $1 \mu\text{M}$ Ca^{2+} no additional increase in F was observed, while a maximal increase at 5 min after addition of ionomycin was obtained at $40 \mu\text{M}$ Ca^{2+} . Halfmaximal increase in F was obtained at Ca^{2+} levels around $20 \mu\text{M}$. This value is very similar to that at which halfmaximal calpain activity and flipflop of phospholipids has been observed in platelets [664].

Changes in fluorescence intensity of TMA-DPH do not necessarily indicate flipflop of

Fig. 4. Temporary character of flipsites in activated platelets. (A) Labeling of ionomycin-activated platelets with TMA-DPH, one minute after (a) or before (b) activation. Arrow indicates addition of TMA-DPH (a) or ionomycin (b). (B) Labeling of thrombin-activated platelets with TMA-DPH, one minute after (a) or before (b) activation. Arrow indicates addition of TMA-DPH (a) or thrombin (b). (C) Labeling of ionomycin-activated platelets with TMA-DPH (arrow) at different times after activation, indicated in seconds by the numbers at the end of the recordings. Experimental details are described in the methods. Data shown are representative for 4 experiments.

TMA-DPH. To confirm that the second increase in F upon addition of ionomycin to Ca^{2+} -buffered platelets is due to transbilayer movement we determined the accompanying changes in lifetime, assuming, analogous to ionomycin-activated platelets, that rapid transbilayer movement of TMA-DPH in platelets is accompanied by a loss of transbilayer fluidity gradient, resulting in a drop in fluorescence lifetime. Lifetimes were determined by phase-modulation fluorimetry before and 5 min after addition of ionomycin to Ca^{2+} -buffered platelets. It was found

that the lifetime of TMA-DPH decreased with a similar Ca^{2+} -dependence as the secondary increase in F (Fig. 5B), with a halfmaximal decrease around $20 \mu\text{M}$ free Ca^{2+} . These findings suggest that the Ca^{2+} -dependent increase in F is due to transbilayer movement of TMA-DPH.

Ca^{2+} -dependent generation of flipsites in fluoride-treated platelets.

The Ca^{2+} - and time-dependence of rapid flipflop of TMA-DPH in ionomycin-activated platelets are similar as found for calpain activity [664], suggesting a relation between cytoskeletal degradation and generation of flipsites. Such a relation would have major mechanistic implications. Therefore, we further explored this suggestion, employing fluoride-treated platelets, which have been used before to demonstrate a relationship between calpain activity and transbilayer movement of phospholipids [663]. Platelets were treated with fluoride for two hours, during which the permeability of the plasma membrane towards Ca^{2+} is transiently enhanced. At several time points during incubation with fluoride, subsamples were labeled with TMA-DPH and extracellular Ca^{2+} was added to stimulate calpain to a level dependent on the prevailing Ca^{2+} permeability.

Fluoride-treated platelets displayed both a Ca^{2+} -independent and Ca^{2+} -dependent increase in fluorescence intensity. The Ca^{2+} -independent increase occurred during the first 15 min (Fig. 6A), and can be explained by the release reaction which follows a similar time course during fluoride-treatment [663]. After 15 min the Ca^{2+} -independent rise in F does not change anymore. The Ca^{2+} -dependent increase in F varies during incubation with fluoride: during fluoride treatment its importance increases and around 30 min fluoride treatment the increase is about 50% relative to Ca^{2+} -independent F , while after 90 min fluoride treatment addition of Ca^{2+} causes an increase of about 10% (Fig. 6A). This transient capacity of extracellular Ca^{2+} to induce an increase in F of fluoride-treated platelets labeled with TMA-DPH resembles the transient increase in Ca^{2+} permeability very well [663].

To investigate whether the Ca^{2+} -dependent increase in F is caused by transbilayer

Fig. 5. Ca^{2+} -dependence of flipflop of TMA-DPH during activation of platelets with Ca^{2+} -ionophore. (A) Changes in fluorescence intensity of TMA-DPH after addition of ionomycin (arrow) to platelets in the presence of various HEDTA/ Ca^{2+} buffers. The extracellular free Ca^{2+} concentration is indicated in μM at the end of the individual recordings. Data shown are representative for 5 experiments. (B) Changes in lifetime of TMA-DPH, as determined by phase-modulation fluorimetry (τ_p), after platelet activation by ionomycin in the presence of Ca^{2+} buffers. Lifetimes were determined two min after addition of TMA-DPH but one min before addition of ionomycin (●), and 5 min after addition of ionomycin to the same sample (▲). Data shown are representative for 3 experiments.

movement of TMA-DPH, we determined the accompanying changes in lifetime of TMA-DPH. It was found that addition of Ca^{2+} to fluoride-treated platelets caused a decrease in lifetime of TMA-DPH as measured by phase-modulation fluorimetry (Fig. 6B). This decrease was maximal around 30 min of incubation with fluoride, where the lifetime was reduced from 6.05 to 5.2 ns, which is comparable to the decrease observed in ionomycin-activated platelets (Fig. 5B). Similar to Ca^{2+} -dependent changes in F in fluoride-treated platelets, the decrease in lifetime became progressively smaller upon prolonged incubation with fluoride. From the parallel time course of Ca^{2+} -dependent increase in F and decrease in lifetime, it is con-

cluded that fluoride-treated platelets transiently exhibit Ca^{2+} -inducible flipsites.

DISCUSSION

Transbilayer movement of TMA-DPH over the platelet plasma membrane appeared to be sensitive to changes in membrane organization found to occur during platelet activation, and a detailed insight in different properties of the flipsites, like their time- and Ca^{2+} -dependence could be obtained. In addition, time-resolved fluorimetry revealed changes in membrane order as a result of the generation of flipsites during platelet activation. Throughout the investigations platelet activation by thrombin and by ionomycin were compared, because of their different potency to induce flipflop of PS in the platelet plasma membrane as reflected by generation of procoagulant activity: thrombin is hardly able to induce flipflop of PS, while the Ca^{2+} ionophore is the most potent stimulator known [50, 124, 664]. In accordance with their different potency in generating platelet procoagulant activity, major differences between thrombin and ionomycin were observed with respect to generation of flipsites.

Stimulation of washed human platelets by thrombin increased F of TMA-DPH, which can be directly interpreted as an increase in membrane-bound TMA-DPH, considering the absence of any change in lifetime. This increase in membrane-bound TMA-DPH has been clearly correlated with the release reaction [345]. Stimulation of platelets by thrombin was not accompanied by increased transbilayer movement of TMA-DPH. This is a surprising result for a relatively small and aspecific membrane probe, since it was expected that during fusion of granules with the plasma membrane flipflop of TMA-DPH would be enhanced due to disturbances of the lipid bilayer structure which necessarily occur during fusion. It is conceivable that actual fusion of membranes is too rapid to allow transbilayer movement of TMA-DPH, which will be limited by the lateral diffusion rate, or that fusion involves too low a percentage of the total membrane to be detectable.

Stimulation of platelets by thrombin increased lipid order as sensed by TMA-DPH,

Fig. 6. Ca^{2+} -dependent generation of flipsites in fluoride-treated platelets. (A) Changes in fluorescence intensity of TMA-DPH during incubation of platelets with fluoride, before and after addition of Ca^{2+} (arrow). Incubation time with fluoride is indicated in min at the end of the individual tracings. (B) Lifetime of TMA-DPH, as determined by phase-modulation fluorimetry (τ_p), in fluoride-treated platelets before (●) and after (▲) addition of Ca^{2+} . Experimental details are described in the methods. Data shown are representative for 3 experiments.

while the lifetime of TMA-DPH was not changed. This is an unexpected result, since fusion with granules, which have a low cholesterol content [243, 556], would be expected to decrease τ_s in the platelet plasma membrane in view of the liquid-crystalline state of the plasma membrane at 37°C [623]. Increased rigidity of the platelet plasma membrane upon activation by thrombin has been observed frequently, with various membrane probes [345, 450, 451, 613]. The increase in steady-state anisotropy of TMA-DPH in thrombin-activated platelets has been related to shape-change, and more specifically with the accompanying cytoskeletal reorganization [345]. However, it can be questioned whether the intracellular compartment is responsible for changes in lipid order as sensed by TMA-DPH, since TMA-DPH is confined to the outer leaflet of the plasma membrane in thrombin-activated platelets.

Generally, the most important membrane components which affect the fluorescence lifetime and anisotropy of TMA-DPH are SphM, cholesterol and proteins [chapter 5, Tables I and II]. The SphM content of the outer monolayer of the platelet plasma membrane is not affected by stimulation by thrombin [chapter 1, Table II; 50], while any change in cholesterol content is likely to be a reduction due to fusion with granular membranes. Therefore, it is suggested that proteins are responsible for the observed increase in τ_s , and not changes in the content of sphingomyelin or cholesterol in the outer monolayer of the plasma membrane. Using a variety of fluorescent probes, Kowalska and Cierniewski [336] demonstrated that binding of fibrinogen to ADP-activated platelets increased lipid order. Thus, we suggest that binding of fibrinogen, and possibly of released adhesive proteins like thrombospondin and fibronectin, to the surface of activated platelets is responsible for the increase in lipid order of the outer monolayer of the platelet plasma membrane observed after stimulation by thrombin.

Stimulation of platelets by ionomycin dramatically changed the lipid organization of the platelet plasma membrane. During activation by ionomycin TMA-DPH becomes subject to massive transbilayer movement. Such rapid and abundant flipflop most likely occurs along local disturbances of the lipid bilayer structure (flipsites), though direct or indirect involvement of membrane proteins cannot be excluded. Within one minute after activation by ionomycin these

flipsites disappear. The time scale of rapid flipflop of TMA-DPH is similar to that of rapid flipflop of PS during generation of platelet procoagulant activity by the Ca^{2+} ionophore A23187 [664]. Ionomycin was equally potent as A23187 in inducing flipflop of PS over the platelet plasma membrane as judged from dose-response relationships (unpublished results). Thus, it is suggested that during stimulation of platelets by ionomycin flipsites are generated, which provide a bidirectional means of transport, explaining the quick loss of phospholipid transbilayer asymmetry and the rapid flipflop of TMA-DPH.

It may be argued that incorporation of ionomycin into the plasma membrane can be responsible for rapid transbilayer movement by directly disturbing the lipid bilayer structure. This possibility can be excluded for the following reasons: (1) addition of ionomycin to platelet lipid vesicles increased lipid order, (2) continuous presence of ionomycin can not explain the temporary character of the flipsites, and (3) the effect of ionomycin on lipid order was independent of Ca^{2+} , unlike the generation of flipsites.

Stimulation of platelets by ionomycin results in a loss of the transbilayer fluidity gradient. This can be explained by a randomization of lipids. Phospholipids randomize as a result of stimulation by a Ca^{2+} ionophore [50]. The loss of cholesterol transbilayer asymmetry as well, as inferred from the loss of the fluidity gradient, suggests a relation between the distribution of phospholipids and cholesterol. The preferential binding of cholesterol to SphM [158] could explain both the maintenance of cholesterol transbilayer asymmetry in unstimulated and thrombin-activated platelets, and the loss of this asymmetry in ionomycin-activated platelets.

Ionomycin-activated platelets display higher fliprates of TMA-DPH than unstimulated platelets, though still an order of magnitude lower than *during* stimulation by ionomycin. Transbilayer movement is influenced by the cholesterol content of the membrane: more cholesterol decreases the fliprate [40, 424]. Apparently, the low fluidity of the outer monolayer in unstimulated platelets gives rise to low fliprates, which are considerably enhanced in ionomycin-activated platelets by the increase in fluidity of the outer leaflet of the plasma membrane.

Comparison of ionomycin-activated platelets with platelet lipid vesicles reveals that the

motional properties of TMA-DPH are similar in both membrane systems (Table I). In addition, sonicated platelets show a decreased lipid order upon previous stimulation by ionomycin, relative to unstimulated platelets and thrombin-activated platelets. These findings suggest that the effect of proteins on platelet membrane fluidity is lost upon stimulation by ionomycin. In platelets treated with a Ca^{2+} ionophore, massive degradation of cytoskeletal proteins takes place [124, 664]. Therefore, it is suggested that proteolysis by calpain abolishes the effect of membrane proteins on lipid order in the platelet membranes. This suggestion implicitly assumes that platelet cytoskeletal proteins interact with the lipid bilayer. Indeed, recent binding studies in our laboratory show that the platelet cytoskeleton (obtained as Triton insoluble residue) has a preferential affinity for PS (Comfurius et al., unpublished results).

The experiments with thrombin- and ionomycin-activated platelets suggest a relationship between rapid flipflop of TMA-DPH, loss of transbilayer lipid asymmetry, and cytoskeletal degradation by calpain during stimulation of platelet procoagulant activity by ionomycin. Such a relation would strongly delineate mechanistic models explaining rapid flipflop of PS during platelet activation. Additional support for such a relation was obtained by examining the Ca^{2+} -dependence of generation of flipsites during platelet activation by ionomycin, and by employing fluoride-treated platelets.

Ca^{2+} titration of the generation of flipsites in intact platelets showed a halfmaximal transbilayer movement of TMA-DPH at about $20 \mu\text{M}$ free Ca^{2+} , which is very similar to the Ca^{2+} -dependence of PS-flipflop and calpain activity measured under the same conditions [664]. Flipflop of TMA-DPH is inferred from the combined increase in F and decrease in lifetime. This seems justified for the following reasons: either the decrease in lifetime is due to penetration of TMA-DPH into the platelet, or may reflect a change in the lipid composition of the outer leaflet of the plasma membrane, as seen in ionomycin-activated platelets. Both possibilities involve transbilayer movement. Ca^{2+} titration of flipflop of TMA-DPH shows that not only the maximal amount of flipflop is Ca^{2+} -dependent but the rate of transbilayer movement as well, suggesting that the rate of flipsite formation is determined by a Ca^{2+} -de-

pendent process and that the flipsites have a short lifetime.

Treatment of platelets with fluoride causes a transient increase in Ca^{2+} permeability of the platelet plasma membrane covering a period of two hours, which was used to demonstrate a relationship between PS-flipflop and calpain activity [663]. Addition of Ca^{2+} to TMA-DPH labeled platelets after various times of fluoride treatment resulted in an increase in F and a decrease in lifetime of TMA-DPH to an extent which followed the transient increase in Ca^{2+} permeability. These findings further support a relation between generation of flipsites, flipflop of PS, and stimulation of calpain activity in platelets.

The findings presented in this paper suggest that flipflop of PS during expression of platelet procoagulant activity occurs via flipsites at which transbilayer movement is greatly enhanced, and that these flipsites are generated only during degradation of cytoskeletal proteins by calpain. A relation between calpain and flipsites can be understood in terms of a calpain-dependent reorganization of the interaction between cytoskeletal proteins and lipids in the cytosolic leaflet of the plasma membrane. It is conceivable that the interaction between cytoskeletal proteins and membrane lipids contributes to the structural organization of the lipid bilayer, and that a loss of this interaction can result in a destabilization leading to a reorganization of the lipid bilayer. This suggestion is in line with general notions about interaction between cytoskeletal proteins and membrane lipids [256, 458], and is corroborated by ample observations relating the structural organization of the cytoskeleton to that of the lipid bilayer of the plasma membrane in erythrocytes [15, 39, 95, 103, 194, 219, 423, 526, 703] and in platelets [28].

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Netherlands) for assistance with the laser fluorimeter.

TABLE I. Fluorescence anisotropy decay parameters of TMA-DPH in various membranes.

membrane preparation	r_0	r	ϕ (ns)	χ^2	DW	Z_{par}	Z_{per}	r_s	S	P	θ_c (°)	$D_{\perp}$ (MHz)
unstimulated platelets	.321 .011	.249 .001	1.08 .14	1.08	1.88	218	259	.258	.884	.343	22.9	34
thrombin-act platelets ^A	.302 .005	.267 .001	1.81 .30	.97	1.99	226	231	.278	.913	.366	19.4	15
thrombin-act platelets ^B	.328 .012	.259 .001	1.04 .14	1.03	1.89	223	264	.267	.903	.353	20.9	30
ionomycin-act platelets ^A	.294 .007	.191 .001	1.33 .10	1.07	1.86	216	234	.217	.775	.294	32.6	50
ionomycin-act platelets ^B	.290 .006	.198 .001	1.35 .10	1.04	1.89	200	244	.223	.788	.301	31.6	47
sonicate unstimulated	.329 .006	.222 .001	1.10 .07	1.05	1.88	181	200	.241	.836	.322	27.6	46
sonicate thrombin-act	.351 .008	.222 .001	1.07 .07	1.08	1.95	260	252	.239	.836	.321	27.6	47
sonicate ionomycin-act	.314 .007	.210 .001	1.27 .09	1.04	1.88	214	231	.234	.813	.314	29.5	44
platelet-lipid Ca^{2+}	.326 .005	.203 .001	1.15 .05	1.09	1.85	205	172	.227	.800	.306	30.7	52
platelet-lipid EGTA	.321 .006	.202 .001	1.10 .06	1.05	1.96	202	239	.225	.796	.303	31.0	56
platelet-lipid ionomycin/ Ca^{2+}	.336 .007	.208 .001	1.04 .06	1.06	1.87	180	233	.229	.808	.308	30.1	56
platelet-lipid ionomycin/EGTA	.312 .005	.206 .001	1.28 .07	1.02	1.90	212	256	.231	.804	.310	30.3	46

Fluorescence anisotropy decay was analysed with a single exponential and a residual anisotropy, as described in the methods, starting at channel 54 (emission peak at channel 26, total number of channels is 1024, time-equivalence per channel was 32.5 ps). Standard deviations of fitted parameters are indicated underneath. Fit quality can be judged from χ^2 , the Durbin-Watson parameter (DW), and the number of zero passages in the autocorrelation function in the parallel (Z_{par}) and perpendicular (Z_{per}) direction. Activated platelets were either labeled 2 min after (A) or 2 min before (B) activation. In all cases, measurements were performed a few minutes after labeling. For further details is referred to the methods. Data shown are representative for at least 3 independent determinations.

TABLE II. Biexponential fluorescence decay parameters of TMA-DPH in various membranes.

membrane preparation	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle \tau \rangle$ (ns)	χ^2	DW	ZP
unstimulated platelets	.238 .004	2.39 .08	.762 .005	7.35 .02	6.89	1.15	1.88	231
thrombin-act platelets ^A	.232 .004	2.52 .07	.768 .015	7.28 .02	6.83	1.04	2.00	224
thrombin-act platelets ^B	.245 .004	2.45 .07	.755 .005	7.18 .02	6.71	1.06	1.78	222
ionomycin-act platelets ^A	.350 .005	2.29 .05	.650 .006	5.73 .02	5.12	1.16	1.74	197
ionomycin-act platelets ^B	.345 .004	2.31 .04	.655 .005	5.69 .01	5.10	1.08	1.88	204
sonicated unstimulated	.388 .003	2.23 .03	.612 .004	6.14 .01	5.41	1.21	1.69	170
sonicate thrombin-act	.352 .005	2.57 .05	.648 .007	6.05 .05	5.40	1.10	1.95	236
sonicate ionomycin-act	.354 .004	2.20 .04	.646 .005	5.90 .02	5.27	1.08	1.84	233
platelet-lipid Ca ²⁺	.382 .004	2.32 .03	.618 .005	5.69 .01	5.01	1.19	1.78	185
platelet-lipid EGTA	.369 .005	2.31 .04	.631 .005	5.66 .02	5.01	1.08	1.98	176
platelet-lipid ionomycin/Ca ²⁺	.390 .004	2.25 .03	.610 .005	5.70 .02	5.01	1.18	1.72	137
platelet-lipid ionomycin/EGTA	.362 .004	2.29 .04	.638 .005	5.72 .02	5.08	1.04	1.78	178

Biexponential analysis of fluorescence decay was performed from channel 54 (emission-peak at channel 28). ZP: zero passages in the autocorrelation function. Standard deviations of fitted parameters are indicated underneath. Further details are described in Table I and the methods. Data shown are representative for at least 3 separate determinations.

TABLE III. Tetraexponential fluorescence decay parameters of TMA-DPH in various membranes.

membrane preparation	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	α_4	τ_4 (ns)	$\langle \tau \rangle$ (ns)	χ^2	DW	ZP
unstimulated platelets	.964 .004	.038	.004 .001	1.35 .21	.008 .001	4.15 .50	.024 .001	7.59 .09	5.96	1.18	1.95	267
thrombin-act platelets ^A	.944 .003	.038	.004 .002	1.33 .32	.011 .001	3.35 .42	.041 .001	7.37 .06	6.21	1.31	1.80	208
thrombin-act platelets ^B	.937 .004	.038	.007 .001	1.18 .17	.014 .001	3.76 .42	.043 .002	7.36 .08	6.15	1.08	1.86	239
ionomycin-act platelets ^A	.887 .006	.038	.019 .001	0.83 .06	.040 .002	3.36 .16	.055 .003	6.02 .07	4.78	1.24	1.75	214
ionomycin-act platelets ^B	.840 .006	.038	.027 .002	1.08 .07	.055 .004	3.51 .18	.079 .005	5.97 .07	4.86	0.99	2.06	261
sonicated unstimulated	.817 .006	.038	.031 .002	0.99 .07	.062 .002	3.25 .14	.090 .003	6.41 .05	5.21	1.12	1.85	245
sonicate thrombin-act	.813 .008	.038	.019 .004	1.32 .19	.067 .003	3.46 .24	.108 .006	6.28 .07	5.23	1.10	1.98	263
sonicate ionomycin-act	.829 .008	.038	.029 .004	1.26 .13	.054 .004	3.47 .31	.088 .007	6.17 .09	5.07	1.03	1.95	268
platelet-lipid Ca^{2+}	.504 .014	.038	.078 .004	0.81 .05	.184 .006	3.19 .11	.235 .009	5.94 .05	4.95	1.10	1.96	276
platelet-lipid EGTA	.342 .019	.038	.094 .005	0.75 .06	.232 .007	3.05 .12	.332 .012	5.86 .05	4.97	1.11	1.96	239
platelet-lipid ionomycin/ Ca^{2+}	.405 .017	.038	.098 .005	0.88 .06	.218 .007	3.16 .11	.279 .010	5.96 .04	4.96	1.10	1.86	235
platelet-lipid ionomycin/EGTA	.306 .020	.038	.099 .007	0.79 .07	.238 .008	3.07 .14	.358 .014	5.91 .05	5.05	1.06	1.81	229

Tetraexponential analysis started right at the emission peak, with τ_1 fixed at 38 ps. Standard deviations of fitted parameters are indicated underneath. Further details are described in Table I and the methods. Data shown are representative for at least 3 independent determinations.

CHAPTER 7

GENERAL DISCUSSION

CELLULAR MECHANISMS OF PLATELET PROCOAGULANT ACTIVITY

One of the major goals throughout the work presented in this thesis was to delineate the chain of cellular processes in platelets, from ligand-receptor interaction towards trans-bilayer movement (flipflop) of phosphatidylserine (PS) in the plasma membrane, rendering the platelet procoagulant. Here, an attempt will be made to highlight the key processes for the expression of platelet procoagulant activity.

A central role in the stimulation of platelet procoagulant activity is attributed to calpain. This is suggested by the ample correlations between calpain activity and procoagulant activity with respect to the time course of their development, their dose-response relationships, and their Ca^{2+} -dependence (chapter 1 and 3). Most convincing, however, is the direct relation between calpain activity and procoagulant activity observed by the effect of leupeptin on both activities in fluoride-treated platelets (chapter 3). It is noteworthy that various unpublished observations further support this relation: incubation at 45°C, depletion of metabolic ATP, and hypo-osmotic treatment, all increase platelet procoagulant activity accompanied by cytoskeletal degradation by calpain.

An intriguing question, pertinent to the evaluation of the importance of calpain in the expression of platelet procoagulant activity, is whether other cellular processes are also in-

involved in the flipflop of PS. From the inability of thrombin to significantly increase platelet procoagulant activity beyond control levels (chapter 1), it can be concluded that normal routes of stimulus-response coupling in platelets are not sufficient to evoke flipflop of PS. This is also suggested by the appearance of procoagulant activity in fluoride-treated platelets, which is delayed with respect to aggregation and release (chapter 3). The ability of the local anesthetic dibucaine to render platelets procoagulant, suggests that phospholipase A₂ and phospholipase C dependent pathways are not necessarily involved in the processes leading to flipflop of PS, in view of the inhibition of both phospholipases and C-kinase by dibucaine [196, 274, 659, 660]. Thus, it is concluded that normal pathways of platelet stimulation are neither sufficient nor obligatory for the expression of platelet procoagulant activity. It may well be, however, that phospholipase C and phospholipase A₂ dependent pathways play a facilitatory role in the generation of procoagulant activity under physiological conditions.

From chapters 2 and 3 it is obvious that elevation of cytosolic Ca^{2+} is an important step in the stimulation of calpain. The Ca^{2+} titrations of unstimulated platelets suggest that, if Ca^{2+} would be the sole mediator, during stimulation of platelets by the combined action

of thrombin and collagen, cytosolic Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) of about $10 \mu\text{M}$ would have to be reached, to explain the level of prothrombinase activity and cytoskeletal degradation (chapter 2). In general, $[\text{Ca}^{2+}]_i$ increases upon platelet activation towards 1 to $2 \mu\text{M}$ as measured by fluorescent Ca^{2+} chelators [263; chapter 1]. Thus, it may well be that expression of platelet procoagulant activity is dependent on higher $[\text{Ca}^{2+}]_i$ than other Ca^{2+} -dependent platelet processes. It has been argued in chapter 2 that the requirement for high Ca^{2+} levels can explain the necessity to use both thrombin and collagen to stimulate platelet procoagulant activity most significantly.

At present this suggestion is investigated in more detail by comparing $[\text{Ca}^{2+}]_i$ in Fura2-loaded platelets, reached upon stimulation by collagen and thrombin, either alone or in combination. Preliminary results are shown in Fig. 1. Stimulation of Fura2-labeled human platelets by thrombin results in a very fast increase in $[\text{Ca}^{2+}]_i$ reaching a maximal value after 8 sec, followed by a gradual decline. This is in agreement with other observations on $[\text{Ca}^{2+}]_i$ after stimulation by thrombin of Fura2-labeled platelets [263, 509, 525] and Quin2-labeled platelets [13, 60, 264, 303, 535, 641, 680]. Stimulation of platelets by collagen induced a small rapid increase in fluorescence, which was found to be due to collagen itself, followed by a gradual increase in $[\text{Ca}^{2+}]_i$ towards a maximal level. The delayed rise of $[\text{Ca}^{2+}]_i$ upon stimulation by collagen has also been observed with Fura2-loaded platelets [510], Quin2-labeled platelets [14, 449] and aequorin-loaded platelets [448, 680]. Stimulation of platelets by the combined action of thrombin and collagen shows a rise in $[\text{Ca}^{2+}]_i$ that seems the sum of both agonists: a rapid rise to high levels followed by a gradual increase reaching a maximal level after about one minute. Clearly, the maximal level is significantly higher than obtained by either agonist alone, though it is still lower than the $[\text{Ca}^{2+}]_i$ reached after stimulation by ionomycin.

Apparently, thrombin and collagen increase $[\text{Ca}^{2+}]_i$ by different mechanisms, resulting in different time courses. Generally, agonist-dependent elevation of $[\text{Ca}^{2+}]_i$ in platelets involves both Ca^{2+} release from the dense tubular system and Ca^{2+} influx via divalent cation channels in the plasma membrane [263]. The importance of Ca^{2+} influx was investi-

gated by stimulation of platelets in the presence of EGTA, which sequesters extracellular Ca^{2+} without interfering with Mg^{2+} -dependent adhesion [558]. It was found that the rise in $[\text{Ca}^{2+}]_i$ by thrombin was greatly dependent on extracellular Ca^{2+} , in that the maximal level was lower and the decline towards basal levels was more rapid than in the presence of extracellular Ca^{2+} (unpublished results). In contrast, the gradual rise of $[\text{Ca}^{2+}]_i$ in collagen stimulated platelets was not dependent on

Fig. 1. Increase in cytosolic Ca^{2+} during stimulation of platelets by various agonists, as observed by Fura2 fluorescence. Washed human platelets were isolated by differential centrifugation basically as described previously [82.BEVE.ECZ] from whole blood drawn from healthy volunteers receiving no medication. Platelets pelleted from platelet rich plasma were resuspended in HEPES buffer (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 6.6, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2 , 5 mM glucose and 0.05% fatty acid-free human serum albumine) to approximately $3 \times 10^8 \text{ ml}^{-1}$ and incubated with $1 \mu\text{M}$ Fura2-AM (Molecular Probes Inc, Eugene OR, U.S.A.) for 30 min at 37°C , following the procedure of Rao et al. [85.RAO-GPW]. After loading, platelets were washed twice in HEPES buffer, and were finally resuspended in HEPES buffer pH 7.4 at room temperature. Fluorescence measurements were performed at 2×10^7 platelets per ml in HEPES buffer pH 7.4 containing 3 mM Ca^{2+} . Fura2-labeled platelets were stimulated by (1) $20 \mu\text{g/ml}$ collagen (Hormon Chemie, Munich, F.R.G.), (2) 4 nM thrombin (isolated and purified from bovine blood according to Rosing et al. [80.ROSLJTG]), (3) collagen plus thrombin, or (4) $0.5 \mu\text{M}$ ionomycin (Calbiochem, U.S.A.) in stirred quartz cuvettes at 37°C under continuous monitoring of fluorescence intensity with a SLM Aminco SPF-500C fluorimeter equipped with a light-tight injection port. Excitation and emission wavelengths were set at 335 and 500 nm with 5 and 20 nm slits, respectively. Agonists were added (indicated by arrow) after setting basal Fura2 fluorescence to zero. Data shown are representative for 6 experiments.

extracellular Ca^{2+} (unpublished results). The effect of EGTA on $[\text{Ca}^{2+}]_i$ during platelet activation by the combined action of thrombin plus collagen resembled that on $[\text{Ca}^{2+}]_i$ during activation by thrombin only: a lower maximal level and a more rapid decline (unpublished results).

The dependence of $[\text{Ca}^{2+}]_i$ in thrombin-activated platelets on extracellular Ca^{2+} is generally observed [13, 263, 264, 303, 395, 599a, 680]. The observation that $[\text{Ca}^{2+}]_i$ in collagen-stimulated platelets is independent of extracellular Ca^{2+} is not in agreement with other findings [14, 263]. Stimulation of platelets by collagen is generally found to be dependent on secondary activation of non-adherent platelets by products of the cyclo-oxygenase pathway (thromboxanes, prostaglandin endoperoxides) formed by platelets adhering to collagen [263, 265a, 510, 536a]. In this way, the delayed increase in $[\text{Ca}^{2+}]_i$ after stimulation by collagen is attributed to Ca^{2+} influx in platelets stimulated by thromboxanes [14, 448, 510]. However, pretreatment of Fura2-loaded platelets with aspirin, which irreversibly inhibits the cyclooxygenase, did not affect the rise in $[\text{Ca}^{2+}]_i$ during stimulation by collagen and thrombin (unpublished results), in agreement with the lack of effect of aspirin on procoagulant activity [734]. The fact that $[\text{Ca}^{2+}]_i$ in collagen-activated platelets, under our conditions, is not dependent on extracellular Ca^{2+} and not dependent on the cyclooxygenase pathway, can be explained by the high collagen to platelet ratio employed under stirring conditions. It is proposed that under these conditions most of the platelets will interact directly with collagen fibers, obviating the use of secondary activation by arachidonic acid products. This is supported by findings of others that, with increasing collagen concentrations, the effect of indomethacin or aspirin decreases [557, 680].

The observations with Fura2-labeled platelets are in line with the notion that Ca^{2+} is a key intracellular mediator of stimulation of platelet procoagulant activity: both calpain activity and procoagulant activity develop only after a delay of 60 to 90 sec in platelets stimulated by the combined action of thrombin and collagen, and require the presence of extracellular Ca^{2+} (chapter 2). In addition, significant calpain- and procoagulant activity in ionophore-activated platelets is observed only at relatively high $[\text{Ca}^{2+}]_i$ of 10 μM and higher (chapter 2).

Thus, it is concluded that maximal physiological stimulation of platelet procoagulant activity requires both collagen and thrombin, because the combination of both agonists generates the highest levels of $[\text{Ca}^{2+}]_i$ necessary for optimal generation of calpain and procoagulant activity.

Though mediators of platelet activation other than calpain may not be obligatory for the expression of platelet procoagulant activity, they may facilitate physiological stimulation of flipflop of PS. This is evident from the measurements with Fura2-labeled platelets (Fig. 1), which show that 1.5 min after stimulation by collagen, $[\text{Ca}^{2+}]_i$ is still below $[\text{Ca}^{2+}]_i$ obtained after stimulation by thrombin. In contrast, collagen is a more potent agonist than thrombin for calpain activity as well as platelet procoagulant activity (chapter 1). Thus, Ca^{2+} -independent mechanisms must operate in collagen-stimulated platelets to lower the Ca^{2+} -dependence of calpain.

In view of the different potencies of thrombin and collagen in stimulation of platelet procoagulant activity, it can be argued that the Ca^{2+} -independent mechanism must be specific for collagen. Thus, it is suggested that collagen can modulate calpain activity independent of Ca^{2+} , either by direct effects on calpain or by indirect effects like substrate modulation. Ca^{2+} -independent modulation of calpain, by its phosphorylation, by modulation of calpastatin, or by the endogenous activator, is complex and still controversial [chapter 1]. On the other hand, phosphorylation of cytoskeletal proteins, as means for modulating their degradation by calpain has been observed on various occasions [512, 645, 723]. At present it is not clear whether phosphorylation of cytoskeletal proteins in collagen-activated platelets is a plausible candidate for modulation of calpain activity, since the phosphorylation pattern is similar to that of thrombin-activated platelets [231]. Another Ca^{2+} -independent mechanism whereby collagen may modulate calpain activity in platelets may be a specific structural reorganization of the membrane skeleton resulting from clustering of transmembrane proteins, imposed by the multi-valent interaction of collagen with transmembrane glycoproteins [360, 560; chapter 1].

Thusfar, only the route leading from platelet stimulation towards calpain activity has been addressed, leaving the route from calpain towards flipflop of PS to discuss in more detail.

The most prominent action of calpain within platelets is degradation of the cytoskeletal proteins filamin, talin and myosin. It is emphasized that the action of calpain is certainly not limited to these proteins (see chapter 1). In addition, the involvement of calpain in expression of platelet procoagulant activity is judged indirectly from cytoskeletal degradation. Thus, it may be that substrates which constitute only a minor fraction of total platelet proteins are relevant for further propagation of the signal for flipflop of PS. However, transbilayer movement in ionophore-activated platelets results in dramatic changes in structure and composition of the plasma membrane which are more likely to result from similarly dramatic changes in cytoskeletal proteins than from hydrolysis of minor platelet proteins. Moreover, cytoskeletal proteins have been implicated in the regulation of phospholipid transbilayer movement in the erythrocyte by direct interaction with the lipid bilayer (chapter 1). Thus, it is concluded that the role of calpain in the expression of platelet procoagulant activity is limited proteolysis of major

actin-binding proteins, which will affect interactions of these proteins with the lipid bilayer of the plasma membrane, providing means for regulation of PS flipflop.

A submembraneous localization of cytoskeletal proteins would be a prerequisite for interactions with membrane lipids. In this respect, the major substrates of calpain in platelets, talin, filamin, and myosin, have been shown to be present in a membrane skeleton preparation, isolated from resting platelets [204, 206] in considerable amounts. In general, membrane skeleton attachment is conducted by protein-protein linkage, involving actin barbed-end capping proteins [136, 229, 684], like talin [123], and actin-crosslinking proteins [136, 229, 684], like filamin [542]. Filamin has been shown to bind to glycoprotein I_b in resting platelets [207, 472, 608, 609]. Anchorage of talin to platelet transmembrane proteins has not been demonstrated, though this is suggested by its presence in a platelet membrane skeleton preparation [204, 206]. In general, talin is found at sites of actin-membrane at-

Fig. 2. The platelet membrane skeleton. Schematic impression based on current literature. The membrane skeleton is attached to membrane proteins and can directly interact with the lipid bilayer. The lipids of the membrane are magnified severalfold with respect to proteins in order to illustrate interactions between cytoskeletal proteins and anionic phospholipids, like phosphatidylserine (filled headgroups).

tachment [33, 86]. In fibroblasts and muscle cells, talin has been shown to bind to a complex of adhesive proteins, possibly belonging to a general class involved in adhesion, constituting a fibronectin receptor [292]. In platelets, the glycoprotein IIb/IIIa complex is implicated as the fibronectin receptor [203, 226, 412a, 486a]. Also in myoblasts calpain activation leads to changed properties of the fibronectin receptor [574]. The role of filamin and talin in mediating a link between the platelet cytoskeleton and transmembrane glycoproteins is in line with observations that local anesthetics decrease the ligand-binding capacity of glycoprotein Ib [119, 490, 608] and the glycoprotein IIb/IIIa complex [120a, 490].

Thus, filamin and talin are implicated in the attachment of an actin-based membrane skeletal network to the platelet plasma membrane, by anchorage to glycoprotein Ib and the glycoprotein IIb/IIIa complex, respectively (Fig. 2). Clearly, such coupling proteins provide suitable means for manifesting interactions with membrane lipids suggested to be involved in the regulation of transbilayer asymmetry of PS. Additional candidates for interaction with

membrane lipids are myosin and α -actinin, both substrates for calpain in platelets. Myosin in platelets is partly present in a submembraneous location [206, 492], while α -actinin has been shown to bind to membrane lipids [85], especially diacylglycerol and fatty acids. Direct interaction between submembraneous cytoskeletal proteins with the lipid bilayer may turn out to be a general phenomenon [458], and is best documented for the erythrocyte cytoskeletal proteins spectrin [64, 112, 113, 398, 404, 425, 426] and band-4.1 [564, 596]. In platelets, the occurrence of direct interactions between membrane lipids and cytoskeletal proteins is demonstrated by recent binding studies at our laboratory, showing that platelet cytoskeletal proteins preferentially bind PS over phosphatidylcholine (PC) (Comfurius et al., unpublished results).

In summary, the following route of cellular mechanisms is suggested to be of major importance for the physiological stimulation of platelet procoagulant activity (Fig. 3). Both Ca^{2+} -dependent and Ca^{2+} -independent processes contribute to the degradation of cytoskeletal proteins by calpain. Thrombin and collagen are the most important stimuli for the Ca^{2+} -dependent and Ca^{2+} -independent route, respectively. The importance of Ca^{2+} is evidenced by the potency of the Ca^{2+} ionophore and may explain the necessity of two strong platelet stimulators to obtain highest physiological stimulation of procoagulant activity. Influx of extracellular Ca^{2+} is needed to obtain sufficiently high levels of $[\text{Ca}^{2+}]_i$ to stimulate calpain. As argued above, phosphorylation of cytoskeletal proteins or structural reorganizations of the membrane skeleton may be involved in the Ca^{2+} -independent pathway. Alternatively, collagen may modulate calpain via its inhibitor (calpastatin) or its activator. Calpain-dependent degradation of cytoskeletal proteins, probably those which interact directly with the lipid bilayer of the platelet plasma membrane, will result in flipflop of PS from the intra- to the extracellular side of the plasma membrane, where PS is responsible for the platelet procoagulant activity. The molecular mechanisms of flipflop of PS will be discussed in the remainder of this chapter.

Fig. 3. Platelet cellular mechanisms involved in the expression of procoagulant activity upon stimulation by collagen and thrombin. Details are described in the text.

MOLECULAR MECHANISMS OF PLATELET PROCOAGULANT ACTIVITY

A second major goal throughout the work presented in this thesis was to describe the molecular mechanisms at the level of the platelet plasma membrane which accompany or cause rapid transbilayer movement of PS. Application of the fluorescent membrane probe TMA-DPH, and the combination of fluorescence intensity and time-resolved fluorescence measurements, provided detailed information about the structural and dynamic properties of the platelet plasma membrane. It was found that platelets exhibit a considerable fluidity gradient over their plasma membrane (chapter 5), and that rapid flipflop of PS was accompanied by rapid transbilayer movement of TMA-DPH (chapter 6), suggesting that rapid flipflop in activated platelets occurs along local defects in the lipid bilayer structure (flipsites). It is the process by which these flipsites are formed and the role of cytoskeletal proteins therein which will be addressed in more detail.

To understand the molecular mechanisms governing the generation of flipsites, it is imperative to understand the molecular organization of the platelet plasma membrane first. The lipid bilayer of the platelet plasma membrane is characterized by an asymmetric distribution of its lipid components (Fig. 4A). SphM and PS are confined to the outer and inner monolayer, respectively, while PC and phosphatidylethanolamine (PE) are enriched in the outer and inner monolayer, respectively. Cholesterol [chapter 5] and polyunsaturated acyl chains [494] are predominantly located in the outer and inner monolayer, respectively. The asymmetric distribution of lipids over the platelet plasma membrane is accompanied by a considerable fluidity gradient over the membrane: the inner leaflet being much more fluid than the outer leaflet. A transmembrane fluidity gradient can be interpreted as a difference in lateral pressure between both monolayers of the membrane [222]. In general, however, membranes with different lateral pressures in their constituent leaflets are not stable

lipid bilayers [154, 164, 549]. Thus, the distribution of lipids over the platelet plasma membrane cannot be reconciled with a stable membrane, implicating involvement of other factors in the maintenance of membrane integrity in unstimulated platelets.

Stabilization of a biological membrane which contains an unstable lipid bilayer most likely is dependent on membrane proteins. These membrane proteins should annihilate the difference in lateral pressure between the two monolayers of the platelet plasma membrane. Therefore, transmembrane proteins are not likely candidates, considering their penetration through both monolayers. Peripheral membrane proteins [34, 161], which do not penetrate deeply into the lipid bilayer could provide a counterbalance for the fluidity gradient, thereby stabilizing the platelet plasma membrane. Shallow penetration of proteins into the lipid bilayer can be accomplished by an amphipathic helix [176, 189, 190, 299a, 370a, 640, 710a], in which hydrophilic and charged amino acid residues are located in the head-group region where they can interact electrostatically with charged headgroups, and in which lipophilic amino acid residues are inserted into the bilayer interior where they interact hydrophobically with the acyl chains [88, 170] (Fig. 4B). Interaction of proteins with lipid monolayers can increase the lateral surface tension, as described for melittin and myelin basic protein [200], an N-terminal fragment of adrenocorticotrophic hormone [665], spectrin [398, 425, 426], apocytochrome c [501a], and plasma apolipoproteins [299].

In cells, cytoskeletal proteins are plausible candidates for a peripheral interaction with the cytosolic monolayer of the plasma membrane [228, 256, 458]. In platelets, a clue about the occurrence of amphipathic helix-like interactions between peripheral membrane proteins and cytosolic membranes may be derived from the motional characteristics of TMA-DPH (chapter 5, Figs. 4 and 5). The rotational diffusion coefficient

cient ($D_{\perp}$) of TMA-DPH follows a different course upon penetration of TMA-DPH into unstimulated platelets than fluorescence lifetime ($\langle\tau\rangle$) and structural order (S): $D_{\perp}$ increases rapidly to a maximal value, while $\langle\tau\rangle$ and S decrease gradually towards a lower limit. Generally, $D_{\perp}$ is inversely related to S [320, 620]. However, the value of $D_{\perp}$ obtained after equilibration of TMA-DPH with platelets (80 MHz) is higher than these in egg-PC vesicles (60 MHz), while the value of S (0.77) can be expected from a cholesterol/phospholipid ratio of 0.6 for the whole platelet (chapter 5, Fig. 6). The discrepancy between S and $D_{\perp}$ of TMA-DPH within platelets can be explained by peripheral interaction of proteins with the mem-

brane: shallow penetration of amino acids in the lipid bilayer would create a discrepancy between the extent of order in the headgroup region, which will dominate S as sensed by TMA-DPH, and the rate of motion of acyl chains, which would not be restricted by the peripheral protein and which determines $D_{\perp}$ of TMA-DPH. An interaction between cytoskeletal proteins and lipid bilayer of the platelet plasma membrane is also suggested by the difference in fluidity between platelet membranes derived from unstimulated platelets and from ionophore-activated platelets, in which calpain has degraded the high-molecular weight actin-binding proteins (chapter 5). Thus, the motional behavior of TMA-DPH within platelets is

Fig. 4. Asymmetric distribution of lipids over the platelet plasma membrane (A), and the stabilization of this distribution by peripheral interaction between cytoskeletal proteins and the cytosolic leaflet (B), involving amphipathic helices. Phospholipids are identified by their headgroups as follows: SphM, ($\square$); PC, ($\circ$); PE, ($\triangle$); PS, ($\bullet$). Intercalated between the phospholipids is cholesterol identified by three chained filled diamonds. The filled rectangles in (B) represent hydrophobic amino-acid residues penetrated into the lipid bilayer.

in line with the notion that amphipathic helix-like interactions between cytoskeletal proteins and the cytosolic leaflet of the plasma membrane occur within platelets.

Before elaborating possible mechanisms by which flippases are generated in the platelet plasma membrane, it seems appropriate to consider the evidence for flippases in more detail. In view of the use of TMA-DPH in the characterization of flippases in the platelet plasma membrane, it can be questioned whether flip-flop of TMA-DPH is representative for flip-flop of phospholipids. Transbilayer movement of TMA-DPH in unstimulated platelets is characterized by an estimated halftime of 60 to 90 min (chapter 5). This flippase can best be compared with flippases of PC, which is not subject to ac-

tive transbilayer movement (chapter 1). The flippase of TMA-DPH does not differ much from that of pyrene-PC in erythrocytes [224] and of short-chain spin-labeled PC in lymphocytes [722]. In general, however, flippases of PC are several times lower than that of TMA-DPH in platelets, like the flippase of PC [218, 413, 643], lyso-PC [40, 423], or short-chain spin-labeled PC [585] in erythrocytes. These results suggest that flippases of TMA-DPH are an overestimation of flippases of phospholipids, but still are in the same order of magnitude.

The flippase of TMA-DPH during generation of platelet procoagulant activity is characterized by an estimated halftime of 15 to 20 sec (chapter 6), which is two orders of magnitude faster than the flippase in unstimulated plate-

Fig. 5. Pore model for flippases in the plasma membrane of activated platelets. The wall of the pore may be lined by hexagonal phase lipid (A, B) or not (C, D). The pore may be lipidic in nature (A, C), or may contain a central transmembrane protein (B, D). Phospholipids are identified by their headgroup symbol, as explained in Fig. 4. Cholesterol is left out of the model for convenience.

lets, and which is similar to the fliprate of PS in ionophore-activated platelets (chapter 2). As already argued in the introduction of chapter 6, active transbilayer movement of TMA-DPH by a translocase must be excluded considering its specificity for the glycerophosphoryl backbone of PE and PS [406]. Exclusion of active transport leaves either passive or facilitated diffusion. Passive diffusion through an intact lipid bilayer could possibly explain the fliprate of TMA-DPH observed in unstimulated platelets ($t_{1/2}$ 60-90 min) and platelets after ionomycin activation ($t_{1/2}$ 10 min), but not for the very high fliprates during ionomycin activation ($t_{1/2}$ 20 sec). Thus, the rapid flipflop of TMA-DPH during platelet activation by ionomycin is explained by transbilayer movement facilitated by flip-sites.

To understand the molecular nature of flip-sites it is illuminating to summarize observations made with model lipid systems. Flipflop of phospholipids in unilamellar lipid vesicles is extremely slow under equilibrium conditions,

i.e. in the absence of any physical or chemical transbilayer asymmetry, with halftimes of several weeks [476, 545]. Flipflop in lipid vesicles can be increased in the following ways: (1) by an asymmetric distribution of phospholipids [339]; (2) by introduction of a transbilayer fluidity gradient [343]; (3) by packing defects at the boundary of gel-liquid crystalline phases [344]; (4) by formation of non-bilayer lipid structures [141, 234]; (5) by introduction of transmembrane proteins [233, 612, 726]. From this summary, it is clear that transbilayer asymmetry of both phospholipids and fluidity over the platelet plasma membrane constitutes an important driving force for transbilayer movement. Nevertheless, passive transbilayer movement in unstimulated platelets is slow, judged by flipflop of PC [623]. Thus, rapid flipflop, as observed during platelet activation by ionomycin, must depend on the generation of structural defects of the lipid bilayer.

Limiting conditions describing the flipsite in activated platelets in more detail can be

derived from findings presented in chapter 6. Generation of flipsites is related to stimulation of calpain, both kinetically and quantitatively. For reasons discussed in the first part of this chapter, involvement of calpain is taken to indicate involvement of membrane skeleton interactions in the generation of flipsites. In addition, flipsites in activated platelets allow bidirectional and aspecific transport of membrane lipids. This is concluded from the inward movement of sphingomyelin (SphM) which accompanies the outward movement of PS (chapter 1), and from the participation of TMA-DPH in rapid flipflop (chapter 6). Moreover, flipsites in activated platelets are transient, as indicated by the disappearance of rapid flipflop within 30 sec after stimulation by ionomycin (chapter 6).

Ultimately, transbilayer movement of lipids along flipsites will be limited by the speed at which these sites can be reached, i.e. by the lateral diffusion coefficient (D) of the lipid in question. In general, D is about 10^{-7} cm²/s for phospholipids [549]. Assuming a globular form with a radius of 1 μ m for platelets, the platelet surface will be about 10^{-7} cm². These data suggest that transbilayer lipid asymmetry over the platelet plasma membrane can be lost on a time scale of seconds, assuming the presence of flipsites, at which the activation energy for transbilayer movement is absent, i.e. at which both leaflets are interconnected. This limiting time scale is close to that of rapid flipflop in ionophore-activated platelets, suggesting that flipsites in activated platelets will have a pore structure which allows direct contact between the two leaflets.

Formation of hydrophilic pores in lipid bilayers is determined by the edge energy, which is directly related to the radius of curvature of the wall of the pore [549]. The energy barrier for pore formation can be overcome by high lateral pressures in the bilayer [549]. In view of these characteristics of pore formation in lipid bilayers, it is suggested that the difference in lateral pressure between the two leaflets of the platelet plasma membrane, as implicated by the fluidity gradient, will drive the formation of pores when the stabilization of the inner leaflet by cytoskeletal proteins is locally disabled by the action of calpain. This model can be extended to include the formation of non-bilayer structures close to the wall of the pore, thereby increasing the radius of curvature and thus reducing the energy for pore formation. Forma-

tion of non-bilayer structures would involve cone-shaped lipids (chapter 1), like PE with unsaturated acyl chains, which is a major constituent of the inner leaflet of the platelet plasma membrane [98, 494]. Formation of non-bilayer structures is in line with findings that the lipids of the inner leaflet of the erythrocyte plasma membrane do not form stable lipid bilayers [291], especially in the presence of Ca²⁺.

A pore model for flipsites in the plasma membrane of activated platelets, based on considerations mentioned above, is shown in Fig. 5. This model contains an annulus of hexagonal phase lipid, which may not be a prerequisite. A further addition to this model could be a transmembrane protein in the central core. The presence of such a protein can lower the energy for pore formation by supplying a hydrophilic transmembrane surface. Involvement of a transmembrane protein would allow regulation of pore formation by conformational changes resulting in exposure or disappearance of the hydrophilic transmembrane surface. In addition, membrane permeability may not be impaired by the presence of a transmembrane protein within the pore, as it most likely will be in the absence of such a protein. To account for the transient nature of flipsites in activated platelets, there must be mechanisms for relaxation of the membrane pore. In the protein-free pore, the local constraint of the fluidity gradient will be removed upon flipflop, and hence the driving force for pore formation is lost leading to closure of the pore. The protein containing pore may be closed by changes in the hydrophilic protein surface, lining the wall of the pore, as well. The pore model for rapid flipflop in activated platelets contains several features which can be tested. The amphipathic helix-like interaction between cytoskeletal proteins and the inner leaflet of the platelet plasma membrane can be demonstrated by appropriate phospholipid photoaffinity labels, or possibly in model systems with lipid monolayers. In addition amphipathic helices can be recognized directly from the primary structure of cytoskeletal proteins [176]. Finally, the occurrence of hydrophilic pores should lead to, at least temporarily, enhanced permeabilities for ions or non-electrolytes.

In conclusion, the kinetics of flipflop in activated platelets suggest transbilayer movement via pores in the plasma membrane. Transmembrane hydrophilic pores may be formed locally

upon relief of the stabilizing interaction between cytoskeletal proteins and the plasma membrane through limited proteolysis by calpain. The driving force for pore formation is the difference in lateral tension between the two leaflets of the plasma membrane, which is related to the fluidity gradient. Formation of non-bilayer structures and the presence of a transmembrane protein in the pore may further facilitate pore formation. Flipflop itself will re-

sult in pore relaxation by elimination of the driving force, the fluidity gradient.

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"It does data processing, word processing and list processing.
Get me some data, some words and some lists."

SUMMARY

Blood platelets play a multifaceted role in the arrest of bleeding upon vessel wall injury. Normally, platelets circulate in the blood stream as quiescent cells. Upon vessel wall injury, platelets become activated: they adhere to the uncovered subendothelial fibrils (adhesion), secrete their granular contents (release reaction), and stick to each other (aggregation) to form a primary hemostatic plug. The primary platelet plug is consolidated by fibrin, which is formed upon initiation of the coagulation cascade, a process which is dramatically accelerated by activated platelets. Finally, contractile mechanisms in platelets lead to clot contraction.

Research in the group of professor Zwaal is concentrated on the acceleration of coagulation by activated human blood platelets. This property of activated platelets is referred to as procoagulant activity, and can easily be measured by the conversion of prothrombin to thrombin by the enzymatic complex of factor X_a and V_a , under conditions at which the procoagulant activity of the platelet is rate limiting. Routinely, purified coagulation factors are used, and the amount of thrombin formed is determined with a chromogenic substrate.

Not all platelet activators are equally potent in stimulation of procoagulant activity. Thrombin is a moderate and collagen is an efficient stimulator of procoagulant activity. Most potent, however, is the combination of collagen and thrombin. The condition of two powerful platelet activators could mean that coagulation is accelerated especially at the site of vessel wall injury.

Previous research showed that negatively charged phospholipids, like phosphatidylserine,

are responsible for the procoagulant activity of the platelet surface. In a quiescent platelet, phospholipids are asymmetrically distributed over the plasma membrane (transmembrane asymmetry), in such a way that phosphatidylserine is virtually confined to the intracellular side of the plasma membrane. This means that phosphatidylserine has to cross the plasma membrane during platelet activation. The molecular and cellular mechanisms involved in the transmembrane movement (flipflop) of phosphatidylserine have been the research subject of this thesis. Initial investigation of platelet protein patterns after gel electrophoresis showed that the potency of various platelet activators to induce procoagulant activity correlated with the extent of degradation of cytoskeletal proteins by a Ca^{2+} -dependent protease (calpain).

The involvement of the cytoskeleton in the expression of platelet procoagulant activity was further investigated by comparing the appearance of phosphatidylserine at the platelet surface with the degradation of cytoskeletal proteins by calpain, at various conditions (chapter 2). The rate of both processes was compared after treatment of platelets by a Ca^{2+} ionophore, the combination collagen and thrombin, and the local anesthetics dibucaine and tetracaine. It was found that on a time-scale of less than one minute, several minutes, and almost one hour, respectively, the generation of procoagulant activity followed an identical course as the generation of calpain activity. The observed similarity suggests that both activities are related. This suggestion was further investigated employing the Ca^{2+} -dependence of calpain. Platelets were activated by a Ca^{2+} iono-

phore, while extracellular Ca^{2+} was controlled with the aid of Ca^{2+} buffers. It was found that the Ca^{2+} -dependence of calpain was almost identical with that of the appearance of procoagulant activity, again indicative of a relation between these processes.

A different approach to investigate the relation between flipflop of phosphatidylserine and degradation of cytoskeletal proteins by calpain was found in the use of fluoride-treated platelets. It appeared that during treatment with fluoride, platelets temporarily increase the permeability of their plasma membrane towards Ca^{2+} . When these fluoride-treated platelets subsequently become exposed to Ca^{2+} , their intracellular Ca^{2+} levels will rise to an extent determined by the current Ca^{2+} permeability of their plasma membranes. Comparison of the generation of procoagulant activity and calpain activity after addition of Ca^{2+} again showed a close resemblance. Convincing evidence for a relation between flipflop of phosphatidylserine and degradation of cytoskeletal proteins was obtained by addition of leupeptin, a specific inhibitor of calpain, during the fluoride treatment. It was found that inhibition of calpain resulted in inhibition of procoagulant activity.

Another clue for a relation between the structure of the cytoskeleton and the asymmetric distribution of phospholipids over the plasma membrane was obtained with the aid of artificial vesicles, pinched off from the platelet plasma membrane (chapter 4). Addition of a synthetic lipid which spontaneously incorporates into the plasma membrane renders the membrane unstable, resulting in extrusion of abundant lipid in the form of a plasma membrane vesicle (spicule). These spicules have lost their transmembrane lipid asymmetry. In addition, the composition of their cytoskeletal proteins has changed. These findings can be explained in terms of a relation between cytoskeletal organization and transmembrane lipid asymmetry.

The molecular mechanisms of transmembrane movement of phosphatidylserine during the generation of platelet procoagulant activity were investigated employing the fluorescent amphiphilic membrane probe TMA-DPH (trimethylammonium-diphenylhexatriene). When TMA-DPH is added to suspended platelets, part of it will immediately incorporate into the outer leaflet of the plasma membrane where it

strongly fluoresces, while most of it will stay in solution where it is not fluorescent.

At first, the fluorescence properties of TMA-DPH in unstimulated platelets were investigated employing advanced fluorimetric techniques (chapter 5). These techniques allow detailed determination of the structural properties of the membrane in the vicinity of TMA-DPH. After addition to unstimulated platelets, TMA-DPH will slowly diffuse into the platelet interior, which is evidenced by an increase in fluorescence due to uptake of TMA-DPH from the solution. Monitoring the fluorescence properties of TMA-DPH during its penetration into the platelet, showed that the structure of intracellular membranes is much more fluid than that of the outer leaflet of the plasma membrane. Comparison of the fluorescence properties of TMA-DPH in various model membranes suggested that an uneven distribution of cholesterol in the platelet is the cause of the observed differences in membrane fluidity. It is concluded that the outer leaflet of the plasma membrane contains about 3 times more cholesterol than the cytosolic leaflet.

Changes in the fluorescence properties of TMA-DPH during stimulation of platelets are described in chapter 6. Stimulation of platelets by a Ca^{2+} ionophore resulted in a rapid increase in fluorescence intensity to the level of lysed platelets. This finding indicates that when phosphatidylserine moves rapidly from the inner side to the outer side of the plasma membrane, TMA-DPH moves rapidly into the platelet. This can be explained by assuming that during generation of platelet procoagulant activity the molecular structure of the plasma membrane becomes locally disturbed. Along these membrane disturbances (flipsites) lipids and lipid-like molecules can rapidly move from either side of the plasma membrane to the other. Further information about the properties of these flipsites was obtained by investigation of their lifetime and Ca^{2+} -dependence. Addition of TMA-DPH 1 minute after the Ca^{2+} ionophore showed that the flipsites were no longer present. Determination of the Ca^{2+} -dependence of the generation of flipsites, measured in the same way as the Ca^{2+} -dependence of cytoskeletal degradation and procoagulant activity, showed a close similarity with the Ca^{2+} -dependence of calpain.

In conclusion, the findings described in this thesis can be explained with the following

model. In unstimulated platelets the transmembrane lipid asymmetry is dependent on a direct interaction of specific cytoskeletal proteins with anionic lipids of the plasma membrane. Stimulation of platelets by the combined action of collagen and thrombin will lead to activation of calpain. Limited proteolysis of cytoskeletal proteins by calpain results in loss of the stabilizing

influence of these proteins on the membrane structure. As a consequence, the membrane lipids will immediately reorganize resulting in a loss of transmembrane lipid asymmetry. Phosphatidylserine, present at the platelet surface, can participate in the acceleration of blood coagulation.

SAMENVATTING

Bloedplaatjes spelen een veelzijdige rol in het stelpen van bloedingen. Normaal gesproken wordt een bloedplaatje in een inactieve toestand meegevoerd door de bloedstroom. Zodra echter een bloedvat beschadigd wordt, raken bloedplaatjes geactiveerd: ze binden aan het blootgelegde bindweefsel (adhesie), scheiden de inhoud van hun granula uit (release reactie), en klonten samen (aggregatie) tot een primaire bloedprop. Tevens kunnen geactiveerde bloedplaatjes de bloedstolling versnellen. Het gevormde fibrine bindt de bloedplaatjes aan elkaar tot een stevige prop (secundaire bloedprop), die samentrekt door een contractie proces in de bloedplaatjes.

Het onderzoek in de groep van professor Zwaal concentreert zich op het versnellen van de bloedstolling door geactiveerde humane bloedplaatjes. Deze eigenschap van geactiveerde bloedplaatjes wordt procoagulant activiteit genoemd, en kan gemakkelijk worden afgeleid uit de snelheid waarmee prothrombine omgezet wordt door factor X_a en V_a in thrombine. Routinematig worden hiervoor gezuiverde stollingsfactoren gebruikt, en wordt de hoeveelheid gevormde thrombine bepaald met een chromogeen substraat.

Niet alle stimulators van bloedplaatjes zijn even goed in staat de procoagulant activiteit op te wekken. Thrombine is een matige en collageen is een goede stimulator van de procoagulant activiteit. De combinatie van collageen met thrombine vormt echter het sterkste fysiologische signaal voor het opwekken van de procoagulant activiteit. De noodzaak voor twee krachtige plaatjes activatoren zou kunnen betekenen dat de vorming van thrombine vooral

versneld wordt door de plaatjes op de plaats van beschadiging van de vaatwand.

In het verleden is gebleken dat negatief geladen fosfolipiden, zoals fosfatidylserine, verantwoordelijk zijn voor de procoagulant activiteit. In een inactief bloedplaatje zijn de fosfolipiden asymmetrisch verdeeld over beide zijden van het plasma membraan (transmembraan asymmetrie), waarbij fosfatidylserine zich vrijwel uitsluitend aan de binnenkant van het plasma membraan bevindt, en dus tijdens de plaatjes activatie door het membraan naar buiten moet bewegen. De moleculaire en cellulaire processen betrokken bij de transmembraan beweging (flipflop) van fosfolipiden zijn onderwerp van het promotie-onderzoek geweest. Onderzoek naar veranderingen in eiwitpatronen met behulp van gelelectroforese, liet in eerste instantie zien dat de mate van procoagulant activiteit, opgewekt door verschillende plaatjes activatoren, correleerde met de mate van afbraak van cytoskelet eiwitten door een Ca^{2+} afhankelijke protease (calpaine) in het bloedplaatje.

De rol van het cytoskelet tijdens het opwekken van procoagulant activiteit in bloedplaatjes is nader onderzocht door het naar buiten komen van fosfatidylserine te vergelijken met de afbraak van cytoskeleteiwitten door calpaine, onder verschillende omstandigheden (hoofdstuk 2). Zo werd de vormingssnelheid van beide processen vergeleken na behandeling van bloedplaatjes met een Ca^{2+} ionofoor, met de combinatie collageen & thrombine, en met lokale anesthetica (dibucaine, tetracaine). Het bleek dat op een tijdschaal van respectievelijk minder dan één minuut, enkele minuten, en bijna één uur, het opkomen van

procoagulant activiteit en calpaine activiteit parallel liep. Deze correlatie suggereert dat beide activiteiten iets met elkaar te maken hebben. Die suggestie werd onderzocht door gebruik te maken van de Ca^{2+} afhankelijkheid van calpaine. Bloedplaatjes werden geactiveerd met een Ca^{2+} ionofoor terwijl extracellulair Ca^{2+} gemanipuleerd werd met behulp van Ca^{2+} buffers. Gevonden werd dat de Ca^{2+} afhankelijkheid van calpaine vrijwel identiek was aan die van het opwekken van procoagulant activiteit, hetgeen opnieuw suggereert dat deze processen met elkaar verbonden zijn.

Een andere manier om de relatie tussen flipflop van fosfatidylserine en afbraak van cytoskeleteiwitten te onderzoeken werd gevonden in bloedplaatjes behandeld met fluoride (hoofdstuk 3). Het bleek dat behandeling met fluoride in afwezigheid van Ca^{2+} , tijdelijk de Ca^{2+} permeabiliteit van het plasma membraan verhoogt. Wanneer deze behandelde bloedplaatjes vervolgens worden blootgesteld aan Ca^{2+} , zal dit Ca^{2+} naar binnen stromen en het plaatje activeren. Onderzoek naar ontstaan van calpaine activiteit en procoagulant activiteit na toevoeging van Ca^{2+} , liet weer zien dat beide parallel liepen. Een sterker bewijs voor een verband tussen cytoskelet afbraak en flipflop van fosfatidylserine werd verkregen door tijdens de fluoride behandeling een remmer van calpaine toe te voegen. Het bleek dat remming van calpaine resulteerde in remming van procoagulant activiteit, een duidelijke indicatie dat afbraak van cytoskelet eiwitten op een of andere manier verantwoordelijk is voor de flipflop van fosfatidylserine.

Een volgende aanwijzing voor een verband tussen de structuur van het cytoskelet en de transmembraan lipiden asymmetrie werd verkregen met behulp van kunstmatige membraan structuren, afgesplitst van het bloedplaatje (hoofdstuk 4). Door toevoegen van synthetisch lipid dat spontaan in het membraan incorporeert, wordt het plasma membraan onstabiel, en zal de overmaat lipid afgesplitst worden in de vorm een klein bolletje omsloten door het afgesplitste membraan (spicule). Het bleek dat in deze spicules de transmembraan lipiden asymmetrie verloren is gegaan. Dit gaat gepaard met een afwijking in de samenstelling van het cytoskelet, met name het eiwit myosine is vrijwel geheel afwezig in de spicules.

Inzicht in het moleculaire mechanisme van transmembraan beweging van fosfolipiden

tijdens het procoagulant worden van bloedplaatjes vereiste een andere aanpak. Gekozen werd voor het gebruik van een fluorescent amfifiel molecuul, TMA-DPH (trimethylammonium-difenylnhexatriën), als membraan sonde. Een klein gedeelte van het TMA-DPH dat toegevoegd wordt aan inactieve bloedplaatjes, wordt zeer snel opgenomen in de buitenste laag van het plasma membraan waar het fluoresceert, terwijl het merendeel in oplossing blijft waar het niet fluoresceert.

In eerste instantie werd TMA-DPH in een niet-gestimuleerd bloedplaatje onderzocht met geavanceerde fluorescentie technieken (hoofdstuk 5). Met deze technieken is het mogelijk uit de fluorescentie eigenschappen van TMA-DPH af te leiden hoe de membraanstructuur in de omgeving van TMA-DPH er uit ziet. Na toevoeging aan bloedplaatjes zal TMA-DPH langzaam naar binnen diffunderen. Dit gaat gepaard met een geleidelijke toename in de fluorescentie intensiteit als gevolg van opname van TMA-DPH uit de oplossing. Door de fluorescentie eigenschappen van TMA-DPH te volgen terwijl het langzaam het bloedplaatje binnendringt, werd gevonden dat de membraanstructuur binnen in het bloedplaatje veel vloeibaarder is dan aan de buitenkant van het plasma membraan. Vergelijking van de fluorescentie eigenschappen van TMA-DPH in verschillende model membranen met bekende samenstelling, suggereerde dat een ongelijkmatige verdeling van cholesterol in het bloedplaatje de oorzaak moest zijn van de gevonden verschillen in vloeibaarheid. Hieruit werd de conclusie getrokken dat de buitenzijde van het plasma membraan ongeveer 3 maal zoveel cholesterol bevat als de binnenzijde.

Onderzoek naar veranderingen in fluorescentie eigenschappen van TMA-DPH als gevolg van bloedplaatjes activatie (hoofdstuk 6), liet zien dat activatie met de Ca^{2+} ionofoor een snelle toename in de fluorescentie intensiteit tot gevolg heeft, tot het niveau van gelyseerde plaatjes die totaal verzadigd zijn met TMA-DPH. Met andere woorden, tijdens activatie van bloedplaatjes door een Ca^{2+} ionofoor, waarbij fosfatidylserine snel over het plasma membrane naar buiten beweegt, beweegt TMA-DPH zich snel naar binnen toe. Dit kan verklaart worden door aan te nemen dat tijdens het opwekken van procoagulant activiteit in bloedplaatjes, de lipiden bilaag structuur van het plasma membraan op moleculair niveau verstoord raakt. Langs deze membraan verstoringen (flipsites)

kunnen dan lipiden snel dwars over het membraan bewegen. Nadere informatie over de eigenschappen van de flipsites werd verkregen door onderzoek naar hun levensduur en de Ca^{2+} afhankelijkheid. Toevoeging van TMA-DPH aan bloedplaatjes één minuut ná de Ca^{2+} ionofoor, liet zien dat de flipsites niet meer aanwezig waren. De Ca^{2+} afhankelijkheid van de generatie van flipsites, gemeten op dezelfde manier als beschreven voor cytoskelet afbraak en procoagulant activiteit, kwam overeen met die van calpaine.

Concluderend, kunnen de hier beschreven waarnemingen met het volgende model verklaart worden. In een inactief bloedplaatje

wordt de transmembraan lipiden asymmetrie mede gehandhaafd door interactie van bepaalde cytoskelet eiwitten met de lipiden laag. Stimulatie van bloedplaatjes door een combinatie van collageen en thrombine leidt tot activering van calpaine, dat bepaalde cytoskelet eiwitten in fragmenten afbreekt. Hierdoor komt de stabiliserende invloed van die cytoskelet eiwitten te vervallen, en zal het membraan zich ogenblikkelijk reorganiseren, resulterend in een verlies van transmembraan lipiden asymmetrie. Het fosfatidylserine dat nu ook aan de buitenkant van het plasma membraan zit zal daar de bloedstolling kunnen versnellen.

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Curriculum vitae

De schrijver van dit proefschrift werd geboren te Maastricht op 16 november 1955. Na het behalen van het diploma Gymnasium-B aan het Stedelijk Lyceum te Maastricht, werd in 1975 aangevangen met de studie scheikunde aan de Rijksuniversiteit Utrecht. In juni 1977 trad hij in het huwelijk met Sylvia Roomberg. In maart 1980 werd het kandidaatsexamen scheikunde met bijvak biologie afgelegd. In januari 1983 werd hij vader van een zoon. In juli 1984 werd het doctoraal examen afgelegd, met als specialisatie biochemie (Dr. R.A. Demel) en als bijvak moleculaire neurobiologie (Prof. Dr. W.H. Gispen). Het onderzoek gedaan tijdens het hoofdvak werd gewaardeerd met de stimuleringsprijs 1983 van de subfaculteit scheikunde. In juli 1984 werd hij tevens vader van een tweeling. Van januari 1985 tot en met december 1987 was hij als onderzoekmedewerker in dienst bij Medigon/NWO, en werd het promotie-onderzoek dat ten grondslag ligt aan dit proefschrift verricht bij de vakgroep Biochemie aan de Rijksuniversiteit Limburg onder leiding van Prof. Dr. R.F.A. Zwaal en Dr. E.M. Bevers. Van februari 1988 tot en met juli 1988 was hij als toegevoegd onderzoeker verbonden aan de Rijksuniversiteit Limburg. Vanaf december 1988 zal hij met behulp van een NATO-science fellowship gaan werken bij Prof. Dr. M.C. Scrutton, verbonden aan het Biochemistry Department van King's College te London, Engeland.