Platelet Signaling

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Abstract This chapter summarizes current ideas about the intracellular signaling that drives platelet responses to vascular injury. After a brief overview of platelet activation intended to place the signaling pathways into context, the first section considers the early events of platelet activation leading up to integrin activation and platelet aggregation. The focus is on the G protein-mediated events utilized by agonists such as thrombin and ADP, and the tyrosine kinase-based signaling triggered by collagen. The second section considers the events that occur after integrin engagement, some of which are dependent on close physical contact between platelets. A third section addresses the regulatory events that help to avoid unprovoked or excessive platelet activation, after which the final section briefly considers individual variations in platelet reactivity and the role of platelet signaling in the innate immune response and embryonic development.

Keywords G proteins • G protein coupled receptors • Signal transduction • Thrombin • Collagen • ADP

1 Overview

The steps in stable platelet plug formation can be summarized in a three stage model as initiation, extension and stabilization, each of which is supported by signaling events within the platelet. *Initiation* occurs when moving platelets become tethered to and activated by collagen/von Willebrand factor (VWF) complexes within the injured vessel wall. This produces a platelet monolayer that supports the subsequent adhesion of activated platelets to each other. *Extension* occurs when additional platelets adhere to the initial monolayer and become activated. Thrombin, ADP and thromboxane A_2 (TxA_2) play an important role in this step, activating platelets via cell surface receptors coupled to heterotrimeric G proteins. Subsequent intracellular signaling activates integrin $\alpha_{IIb}\beta_3$ (glycoprotein (GP) IIb-IIIa in older literature) on the platelet surface, thereby enabling cohesion between platelets. *Stabilization* refers to the late events that help to consolidate the platelet plug and prevent premature disaggregation, in part by amplifying signaling within the platelet. Examples include outside-in signaling through integrins and contact-dependent signaling through receptors whose ligands are located on the surface of adjacent platelets. The net result is a hemostatic plug composed of activated platelets embedded within a cross-linked fibrin mesh, a structure stable enough to withstand the forces generated by flowing blood in the arterial circulation.

This three stage model arises from studies on platelets from individuals with monogenic disorders of platelet function and from mouse models in which genes of interest have been knocked out. However, recent observations suggest that the model is overly simplistic in presenting platelet accumulation after injury as a linear, unstoppable and nonreversible series of events. In fact, there is now ample evidence for spatial as well as temporal heterogeneity within a growing hemostatic plug (Yang et al. 2002; Reininger et al. 2006; Ruggeri et al. 2006; Nesbitt et al. 2009;

Bellido-Martin et al. 2011; Brass et al. 2011). This means that at any given time following injury there are fully activated platelets as well as minimally activated platelets, not all of which will inevitably become fully activated. Furthermore, with the passage of time, incorporated platelets draw closer together and many remain in stable contact with each other. This allows contact-dependent signaling to occur and produces a sheltered environment in which soluble molecules can accumulate. Thus, a more updated view of platelet activation needs to be less ordered than the three stage model, reflect differences in the extent of activation of individual platelets and incorporate the consequences of platelet:platelet interactions in a three dimensional space.

1.1 Molecular Events

Under steady state conditions, platelets circulate in an environment bordered largely by a continuous monolayer of endothelial cells. They move freely, but are quiescent. Once vascular injury has occurred, platelets are principally activated by locally exposed collagen, locally generated thrombin, platelet-derived thromboxane A_2 (TxA_2) and ADP that is either secreted from platelet dense granules or released from damaged cells. VWF serves as an essential accessory molecule. In the pre-injury state, VWF is found in plasma, within the vessel wall and in platelet α-granules. Additional VWF/collagen complexes form as collagen fibrils come into contact with plasma. Circulating erythrocytes facilitate adhesion to collagen by pushing platelets closer to the vessel wall, allowing GP Ibα on the platelet surface to be snared by the VWF A1 domain. Once captured, the drivers for platelet activation include the receptors for collagen (GP VI) and VWF (GP Ibα), thrombin (PAR1 and PAR4), ADP ($P2Y_1$ and $P2Y_{12}$) and thromboxane A_2 (TP) (Fig. 1).

In general terms, agonist-initiated platelet activation begins with the activation of one of the phospholipase C (PLC) isoforms expressed in platelets. By hydrolyzing membrane phosphatidylinositol-4,5-bisphosphate (PIP_2), PLC produces the second messenger inositol-1,4,5-trisphosphate (IP_3) needed to raise the cytosolic Ca^{2+} concentration. This leads to integrin activation via a pathway that currently includes a Ca^{2+}-dependent exchange factor (CalDAG-GEF), a switch (Rap1), an adaptor (RIAM), and proteins that interact directly with the integrin cytosolic domains (kindlin and talin) (Shattil et al. 2010). Which PLC isoform is activated depends on the agonist. Collagen activates PLCγ2 using a mechanism that depends on scaffold molecules and protein tyrosine kinases. Thrombin, ADP and TxA_2 activate PLCβ using G_q as an intermediary.

The rise in the cytosolic Ca^{2+} concentration that is triggered by most platelet agonists is essential for platelet activation. In resting platelets, the cytosolic free Ca^{2+} concentration is maintained at approximately 0.1 μM by limiting Ca^{2+} influx and pumping Ca^{2+} out of the cytosol either out across the plasma membrane or into the dense tubular system. In activated platelets, the Ca^{2+} concentration rises tenfold to >1 μM as Ca^{2+} pours back into the cytosol from two sources. The first

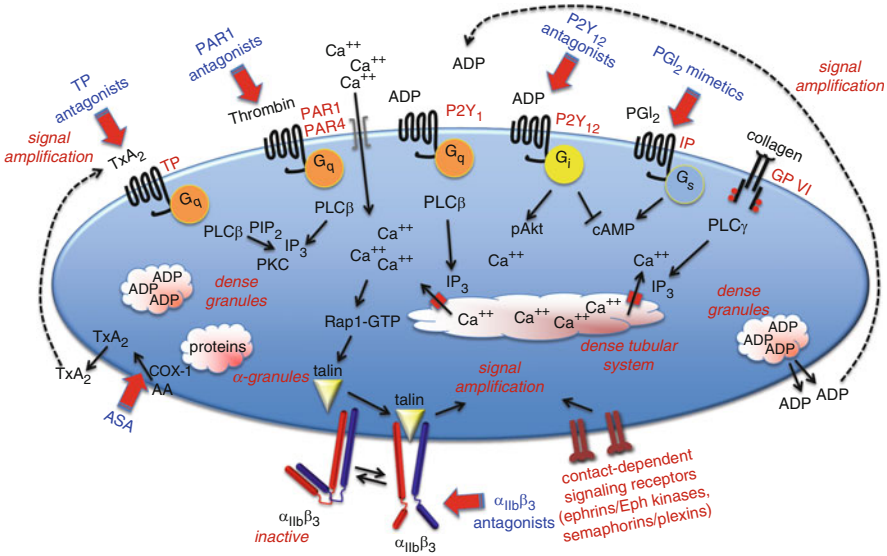

Fig. 1 An overview of some of the pathways that support platelet activation. Targets for antiplatelet agents that are currently in clinical use or in clinical trials are indicated in blue. PLC, phospholipase C; PKC, protein kinase C; IP_3, inositol-1,4,5-trisphosphate; TxA_2, thromboxane A_2: GP, glycoprotein; IP and TP, PGI_2 and TxA_2 receptors

is IP_3-mediated release of Ca^{2+} from the platelet dense tubular system (DTS). The second is Ca^{2+} influx across the platelet plasma membrane, an event triggered when depletion of the DTS Ca^{2+} pool produces a conformational change in STIM1, a protein located in the DTS membrane. This conformational change promotes the binding of STIM1 to Orai1 in the plasma membrane allowing Ca^{2+} entry (Varga-Szabo et al. 2011).

Ultimately, it is the binding of fibrinogen or another bivalent ligand to $\alpha_{IIb}\beta_3$ that enables platelets to stick to each other. Proteins that can substitute for fibrinogen include fibrin, VWF and fibronectin. Average expression levels of $\alpha_{IIb}\beta_3$ range from approximately 50,000 per cell on resting platelets to 80,000 on activated platelets. Mutations in $\alpha_{IIb}\beta_3$ that suppress its expression or function produce a bleeding disorder (Glanzmann's thrombasthenia) because platelets are unable to form stable aggregates. Antiplatelet agents such as Integrilin (eptifibatide) and ReoPro (abciximab) take advantage of this by blocking $\alpha_{IIb}\beta_3$.

2 The Early Events of Platelet Activation

Signaling within platelets begins with activation of receptors on the platelet surface by agonists such as collagen, thrombin, ADP, TxA_2 and epinephrine. With the exception of collagen, each of these works through one or more members of the G

protein coupled receptor superfamily. Some of the properties that are common to G protein coupled receptors (GPCRs) make them particularly well-suited for their tasks in platelets. Most bind their ligands with high affinity and each occupied receptor can activate multiple G proteins, amplifying the initial signal. In general, the GPCRs expressed on the platelet surface are present in low copy number, ranging from a few hundred (epinephrine receptors and $P2Y_1$ ADP receptors) to a few thousand (PAR1) copies per cell. Their duration of signaling is subject to receptor internalization, receptor desensitization and the accelerated inactivation of G proteins by members of the RGS (Regulators of G protein Signaling) family. This multiplicity of mechanisms means that platelet activation can be tightly regulated even at its earliest stages.

Although there is now growing evidence in cells other than platelets that GPCRs can signal by more than one mechanism, the events that have been described downstream of the GPCRs in platelets are mediated by heterotrimeric ($\alpha\beta\gamma$) G proteins. Human platelets express ten members of the G_s, G_i, G_q and G_{12} families. This includes at least one G_s, four G_i (G_{i1}, G_{i2}, G_{i3} and G_z), three G_q family members (G_q, G_{11} and G_{16}) and two G_{12} family members (G_{12} and G_{13}). As will be discussed below, much has been learned about the role of G proteins in platelets through studies on mice in which the genes encoding one or more forms of G_α have been disrupted.

2.1 Categorizing Critical Events

Faced with the profusion of signaling events underlying platelet activation, it can be helpful to divide them into categories. The first category begins with the activation of PLC, which cleaves membrane PIP_2 to produce IP_3 and diacylglycerol (DAG), the second messengers needed to raise the cytosolic Ca^{2+} concentration and activate some of the protein kinase C (PKC) isoforms found in platelets. As already noted, collagen activates PLCγ2 using adaptor molecules and tyrosine kinases; thrombin, ADP and TxA_2 activate PLCβ using G_q and, perhaps, G_i family members. The subsequent increase in cytosolic Ca^{2+} triggers downstream events including integrin activation and TxA_2 formation.

The second category of critical events involve monomeric G proteins in the Rho and Rac families, whose activation trigger the reorganization of the actin cytoskeleton that underlies filopodia and lamellopodia formation. Along with changes in the platelet's circumferential microtubular ring, filopodia and lamellopodia formation are the essence of platelet shape change. When platelets in suspension are activated by soluble agonists, shape change precedes platelet aggregation. Most platelet agonists can trigger shape change, the notable exception being epinephrine. The soluble agonists (thrombin, ADP and TxA_2) that trigger shape change typically act through receptors that are coupled to members of the G_q and G_{12} family.

The third category of critical events includes the suppression of cyclic adenosine monophosphate (cAMP) synthesis by adenylyl cyclase, provided the intracellular

cAMP concentration has been raised above baseline by the action of endothelium-derived PGI_2 and nitric oxide (NO). Inhibition of cAMP formation relieves a block on platelet signaling that otherwise serves to suppress inappropriate platelet activation. The agonists that inhibit cAMP formation in platelets do so by binding to receptors coupled to the G_i family members, especially G_{i2} (ADP and thrombin) and G_z (epinephrine). Suppression of adenylyl cyclase is clearly critical when cAMP levels are elevated. It is less clear that it is necessary under basal cAMP conditions.

The fourth category involves activation of the PI 3-kinase isoforms expressed in platelets, either by G_i family members (PI3Kγ) or by phosphotyrosine-dependent signaling pathways downstream of collagen receptors (PI3Kαβδ). PI 3-kinases phosphorylate PI-4-P and PI-4,5-P_2 to produce PI-3,4-P_2 and PI-3,4,5-P_3. Among the best-described consequence of PI3K activation in platelets is the activation of the protein kinase, Akt. Knockout and inhibitor studies demonstrate that all three Akt isoforms are necessary for normal platelet activation (Chen et al. 2004; Woulfe et al. 2004; O'Brien et al. 2011). Knockout and inhibitor studies also show a G_i/PI3K-dependent mechanism for activating Rap1 (Woulfe et al. 2002; Yang et al. 2002) which, given the clinical utility of $P2Y_{12}$ antagonists as antiplatelet agents, seems to be a mechanism that is important for stabilizing platelet aggregates.

3 G proteins and Their Effectors in Platelets

Platelet agonists are not equally potent. Thrombin provides a robust stimulus for phosphoinositide hydrolysis and causes the largest and fastest increase in cytosolic Ca^{2+}. Collagen and ADP are more dependent on the synthesis and release of TxA_2 to achieve a maximal response. In the case of those whose receptors are GPCRs, potency is determined in part by which G proteins their receptors are coupled to, the number of receptor copies, the efficiency with which they activate the G proteins and the susceptibility of any given receptor/G protein pair to signal suppression.

3.1 G_q, Phosphoinositide Hydrolysis, Cytosolic Ca^{2+} and Integrin Activation

The critical role of G_q in platelets is reflected by the major defect in platelet activation observed in $G_{q\alpha}{}^{-/-}$ platelets (Offermanns et al. 1997). As already noted, agonists like thrombin, TxA_2 and (to a lesser extent) ADP whose receptors are coupled to G_q provide a strong stimulus for phosphoinositide hydrolysis in platelets by activating PLCβ (Fig. 2). Different isoforms of PLCβ can be activated by either G_α or $G_{\beta\gamma}$ (or both). PLCβ-activating α subunits are typically derived from

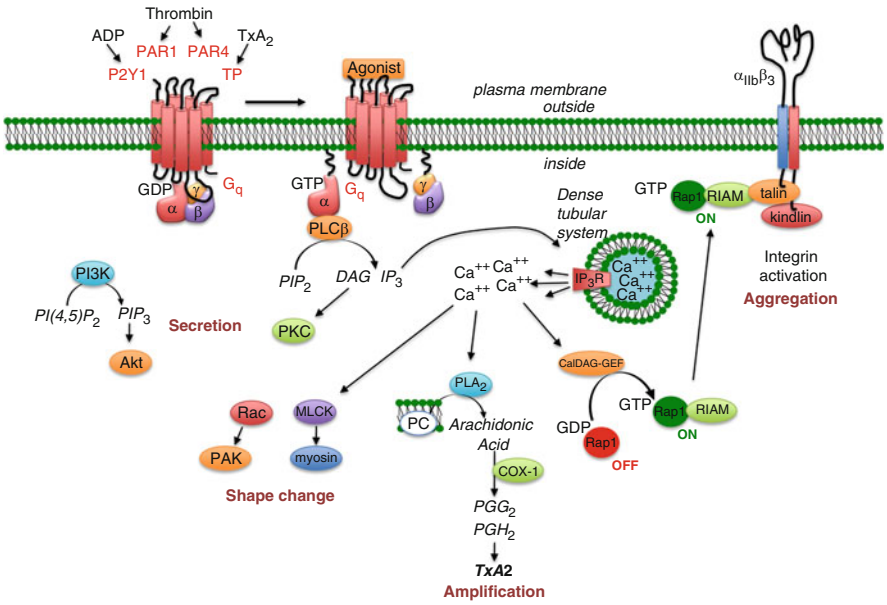

Fig. 2 G_q signaling in platelets. Agonists whose receptors are coupled to G_q are able to activate PLCβ via $G_{q\alpha}$. The potency with which the activation occurs varies with the agonist, with thrombin and TxA_2 providing a stronger stimulus for PLCβ-mediated phosphoinositide hydrolysis than ADP. Thrombin activates two G_q-coupled receptors on human platelets, PAR1 and PAR4, which differ somewhat in the kinetics of PLC activation. Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; COX-1, cyclooxygenase 1; DAG, diacylglycerol; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; IP3 R, IP_3 receptor; MLCK, myosin light chain kinase; PI3K, phosphatidylinositol 3-kinase; PAK, p21-activated kinase; PAR, protease-activated receptor; PG, prostaglandin; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLCβ, phospholipase Cβ; TxA_2, thromboxane A_2

G_q in platelets, but in theory PLC can also be activated by $G_{\beta\gamma}$ derived from G_i family members. Whether this actually occurs in platelets remains to be demonstrated. In a recent study in which $G_{i2\alpha}$ was replaced with a gain of function mutant, there was an increase in G_{i2}-dependent activation of Akt, but no increase in the cytosolic Ca^{2+} response, which suggests that this mechanism is not a major contributor to PLC activation (Signarvic et al. 2010).

Once initiated, the rising Ca^{2+} concentration in activated platelets can trigger integrin activation via the CalDAG-GEF/Rap1/RIAM pathway (Lee et al. 2009; Shattil et al. 2010). This pathway accounts for the ability of Ca^{2+} ionophores to trigger platelet aggregation. Its biological relevance is reflected by the reduction of aggregation in platelets from Rap1 and CalDAG-GEF knockout mice (Crittenden et al. 2004; Chrzanowska-Wodnicka et al. 2005). That the CalDAG-GEF/Rap1/RIAM pathway is not the only way to accomplish integrin activation is reflected by the incomplete reduction of aggregation in $Rap1^{-/-}$ platelets and the ability of PKC

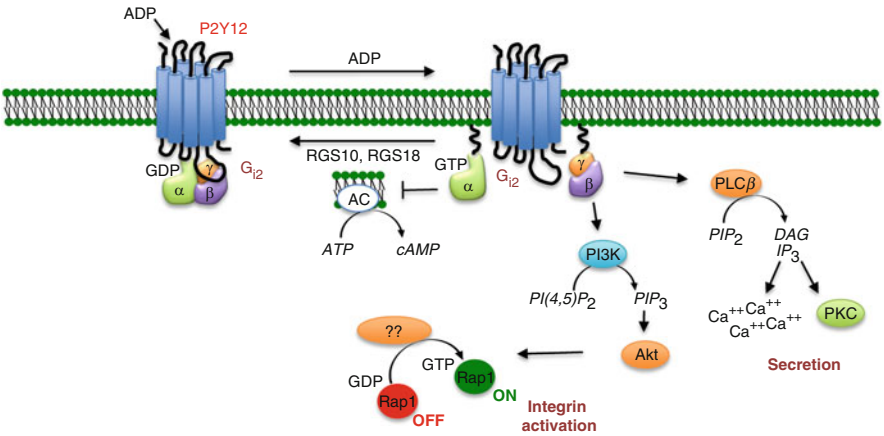

Fig. 3 G_i signaling in platelets. G_{i2} is the predominant G_i family member expressed in human platelets. In addition to inhibiting adenylyl cyclase (alleviating the repressive effects of cAMP), G_{i2} couples $P2Y_{12}$ ADP receptors to PI 3-kinase, Akt phosphorylation and Rap1B activation. Other effectors may exist as well. Abbreviations: AC, adenylyl cyclase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; PI3K, phosphatidylinositol 3-kinase; PLA_2, phospholipase A_2; PLCβ, phospholipase Cβ; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C

activators such as phorbol myristate acetate (PMA) to cause aggregation without causing an increase in cytosolic Ca^{2+} (Shattil and Brass 1987). PKC isoforms phosphorylate multiple cellular proteins on serine and threonine residues. Activation of PKC by PMA is sufficient to cause integrin activation, granule secretion and platelet aggregation in the absence of an increase in cytosolic Ca^{2+}. However, just as the ability of Ca^{2+} ionophores to activate platelets on their own does not fully account for platelet activation in vivo, neither does the response to PMA.

3.2 G_i Family Members, cAMP and PI 3-Kinase

Rising cAMP levels turn off signaling in platelets and an increase in cAMP synthesis is one of the mechanisms by which endothelial cells prevent inappropriate platelet activation. PGI_2 and NO released from endothelial cells cause $G_{s\alpha}$-mediated increases in adenylyl cyclase activity (PGI_2) and inhibit the hydrolysis of cAMP by phosphodiesterases (NO). When added to platelets in vitro, PGI_2 can cause a >10-fold increase in the platelet cAMP concentration, but even relatively small increases in cAMP levels (twofold or less) can impair thrombin responses (Keularts et al. 2000). Platelet agonists such as ADP and epinephrine inhibit PGI_2-stimulated cAMP synthesis by binding to receptors that are coupled to one or more G_i family members (Fig. 3). Disruption of the genes encoding $G_{i2\alpha}$ or $G_{z\alpha}$ causes an

increase in the basal cAMP concentration in mouse platelets (Yang et al. 2002). Conversely, loss of PGI_2 receptor (IP) expression causes a decrease in basal cAMP levels, enhances responses to agonists and predisposes mice to thrombosis in arterial injury models (Murata et al. 1997; Yang et al. 2002).

Although the G_i family members in platelets are most commonly associated with their role in the suppression of cAMP formation, this is not their only role. Neither the defect seen in platelets that are missing $G_{i\alpha}$ family members nor the response to ADP measured in the presence of $P2Y_{12}$ antagonists (Daniel et al. 1999) can be reversed solely by adding inhibitors of adenylyl cyclase. Other downstream effectors for G_i family members in platelets include PI 3-kinase, Src family members and Rap1B (Dorsam et al. 2002; Lova et al. 2002; Woulfe et al. 2002, 2004). The role of Rap1B in integrin activation was discussed in the context of G_q, Ca^{2+} and CalDAG-GEF. PI 3-kinases phosphorylate PI-4-P and PI-4,5-P_2 to produce PI-3,4-P_2 and PI-3,4,5-P_3. Human platelets express the α, β, γ and δ isoforms of PI 3-kinase, each of which is composed of a catalytic subunit and a regulatory subunit. The α, β and δ isoforms are activated by binding to phosphorylated tyrosine residues. The PI3Kγ isoform is activated by $G_{\beta\gamma}$ derived from G_i family members.

Much of what is known about the role of PI 3-kinase in platelets comes from studies with inhibitors such as wortmannin and LY294002, or from studies of gene-deleted mouse platelets (Jackson et al. 2004). Those studies have established that PI3K activation can occur downstream of both G_q and G_i family members and that effectors for PI3K in platelets include the serine/threonine kinase, Akt (Woulfe et al. 2004), and Rap1B (Jackson et al. 2005). Loss of the PI3Kγ isoform causes impaired platelet aggregation (Hirsch et al. 2001). Loss of PI3Kβ impairs Rap1B activation and thrombus formation in vivo, as do PI3Kβ-selective inhibitors (Jackson et al. 2005). Most of the Akt expressed in platelets appears to be the Akt2 isoform, but Akt1 and Akt3 (O'Brien et al. 2011) are present as well. All three appear to contribute to platelet activation, at least in mice. Deletion of the gene encoding Akt2 results in impaired thrombus formation and stability, and inhibits secretion (Woulfe et al. 2004). Loss of Akt1 inhibits platelet aggregation (Chen et al. 2004) and affects vascular integrity (Chen et al. 2005). Loss of Akt3 impairs aggregation and secretion (O'Brien et al. 2011).

3.3 G_q, G_{13} and the Reorganization of the Actin Cytoskeleton

At least two effector pathways are involved in the reorganization of the actin cytoskeleton that accompanies platelet activation: Ca^{2+}-dependent activation of myosin light chain kinase downstream of G_q family members and activation of Rho family members downstream of G_{13} (Fig. 4) (Klages et al. 1999; Offermanns 2001). Several proteins having both G_α-interacting domains and guanine nucleotide exchange factor (GEF) domains can link G_{12} family members to Rho family members, including p115RhoGEF (Fukuhara et al. 2001). With the exception of

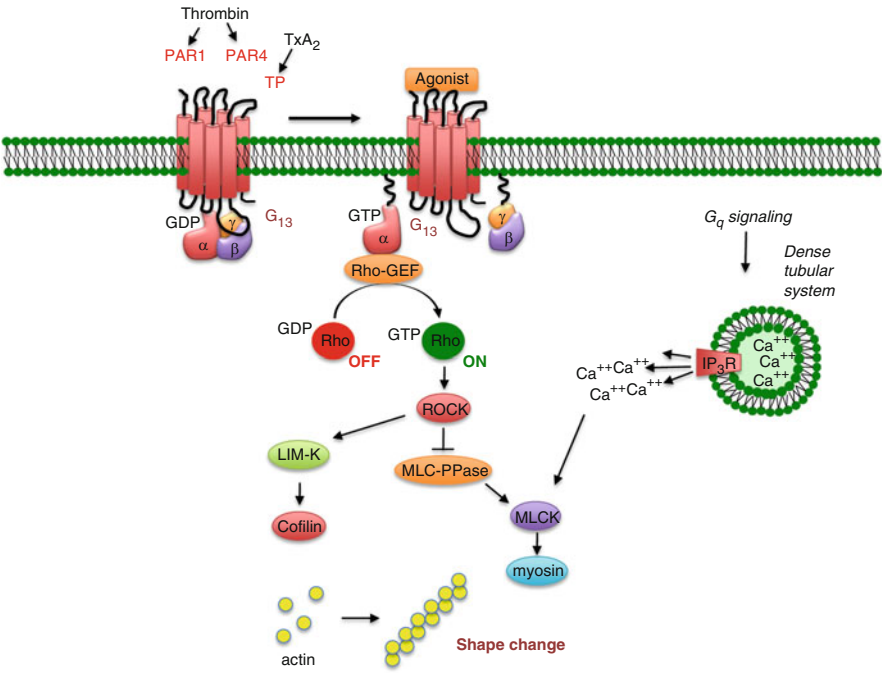

Fig. 4 $G_{12/13}$ signaling in platelets. Agonists whose receptors are coupled to the G_{12} family members expressed in platelets are able to trigger shape change, in part by Rho-dependent activation of kinases that include the Rho-activated kinase, p160 ROCK, and the downstream kinases, MLCK and LIM-K. Although G_{12} and G_{13} are both expressed, based on knockout studies, G_{13} is the dominant G_{12} family member in mouse platelets. Y27632 inhibits p160 ROCK. Abbreviations: GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; IP3 R, receptor for 1,4,5-IP_3; MLCK, myosin light chain kinase; PAR, protease-activated receptor; TxA_2, thromboxane A_2

ADP, shape change persists in platelets from mice that lack $G_{q\alpha}$ but is lost when $G_{13\alpha}$ expression is suppressed, alone or in combination with $G_{12\alpha}$ (Moers et al. 2003, 2004). These results indicate that for most platelet agonists, G_{13} signaling is essential for shape change and that the events underlying shape change are invoked by a combination of G_{13}- and G_q-dependent signals. A combination of inhibitor and genetic approaches suggest that G_{13}-dependent Rho activation leads to shape change via pathways that include the Rho-activated kinase (p160ROCK) and LIM-kinase (Klages et al. 1999; Wilde et al. 2000; Pandey et al. 2005). Activation of these kinases results in phosphorylation of myosin light chain kinase and cofilin, helping to regulate both actin filament formation and myosin. ADP, on the other hand, depends more heavily on G_q-dependent activation of PLC to produce shape change and is able to activate G_{13} only as a consequence of TxA_2 generation; hence ADP-induced shape change is lost when G_q signaling is suppressed.

3.4 Phospholipase A_2, Arachidonate, COX-1 and TxA_2 Production in Platelets

In addition to activating PLC, platelet agonists can activate phospholipase A_2 which cleaves membrane phospholipids, liberating arachidonate from the C2 position of the glycerol backbone (Fig. 2). Arachidonate can be transformed into a very large number of bioactive compounds, but TxA_2 is the key product in platelets. TxA_2 synthesis begins with the cyclooxygenase, COX-1, which forms PGG_2 and PGH_2 from arachidonate in a two step process that is inhibited by aspirin. PGH_2 is then metabolized to TxA_2 by thromboxane synthetase. Evidence suggests that phospholipase A_2 activation in platelets can occur in more than one way. It can clearly happen in response to an increase in cytosolic Ca^{2+} as the addition of a Ca^{2+} ionophore is sufficient to cause phospholipase A_2 activation. There is also evidence that MAPK pathway signaling activates phospholipase A_2, although not necessarily by direct phosphorylation of phospholipase A_2 (Börsch-Haubold et al. 1995, 1999). Once formed, TxA_2 can diffuse out of the platelet, activating nearby receptors in an autocrine or paracrine fashion before it is hydrolyzed to inactive TxB_2. Even though TxA_2 is a potent platelet activator, its half-life in aqueous solution is limited, which has implications for both its duration of action and its impact downstream from a growing thrombus. Studies on TxA_2-induced platelet activation are commonly performed with stable analogs such as U46619.

4 Signaling Responses by Selected Platelet Agonists

4.1 Platelet Activation by Collagen

Four distinct collagen receptors have been identified on human and mouse platelets. Two bind directly to collagen ($\alpha_2\beta_1$ and GP VI); the other two bind to collagen via VWF ($\alpha_{IIb}\beta_3$ and GP Ibα) (Fig. 5). Of these, GP VI is the most potent signaling receptor (Clemetson et al. 1999). The structure of the GP VI extracellular domain places it in the immunoglobulin superfamily. Its ability to generate signals rests on its constitutive association with the ITAM-containing Fc receptor γ-chain (FcRγ). Loss of FcRγ affects collagen signaling in part because of loss of a necessary signaling element and in part because FcRγ is required for GP VI to reach the platelet surface. The $\alpha_2\beta_1$ integrin also appears to be necessary for an optimal interaction with collagen, supporting adhesion to collagen and acting as a source of integrin-dependent signaling after engagement (Keely and Parise 1996; Consonni et al. 2012). However, this appears to require an initial wave of signaling that activates $\alpha_2\beta_1$, much as the fibrinogen receptor, $\alpha_{IIb}\beta_3$ is activated by signaling within platelets. Human platelets with reduced expression of $\alpha_2\beta_1$ have impaired collagen responses, as do mouse platelets that lack β1 integrins when the ability of these platelets to bind to collagen is tested at high shear (Nieswandt et al. 2001; Kuijpers et al. 2003).

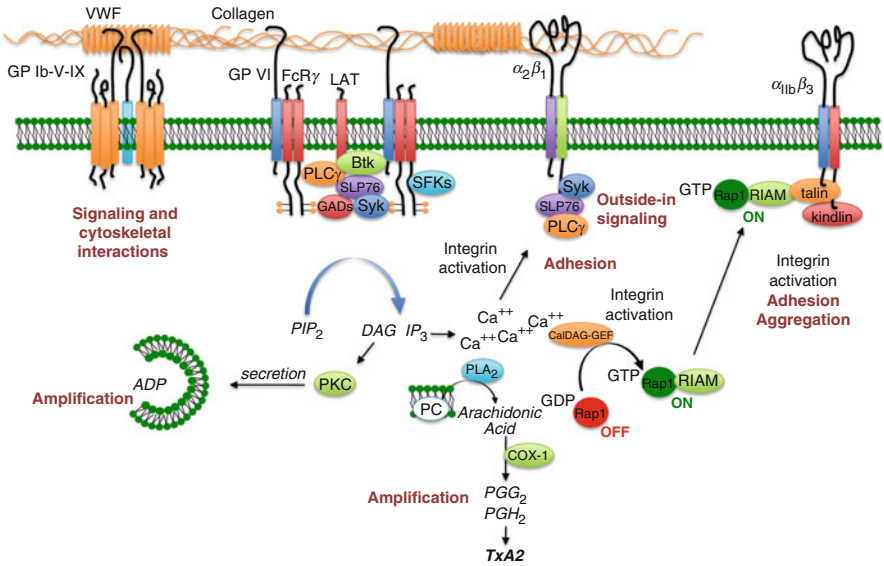

Fig. 5 Platelet activation by collagen. Platelets use several different molecular complexes to support platelet activation by collagen. These include (1) VWF-mediated binding of collagen to the GPIb-IX-V complex and integrin $\alpha_{IIb}\beta_3$, (2) a direct interaction between collagen and both the integrin $\alpha_2\beta_1$ and the GP VI/FcRγ-chain complex. Clustering of GP VI results in the phosphorylation of tyrosine residues in the FcRγ cytoplasmic domain, followed by the binding and activation of the tyrosine kinase, Syk. One consequence of Syk activation is the phosphorylation and activation of phospholipase Cγ, leading to phosphoinositide hydrolysis, secretion of ADP and the production and release of TxA_2

Signaling through GP VI can be studied in isolation with the snake venom protein, convulxin, or with synthetic "collagen-related" peptides (CRP), both of which bind to GP VI, but not to other collagen receptors. According to current models, collagen causes clustering of GP VI. Polymerization of soluble collagen and clustering of GP VI/FcRγ complexes contribute to the lag that is commonly observed when collagen is added to platelets in an aggregometer. This leads to the phosphorylation of FcRγ by Src family tyrosine kinases associated with a proline-rich domain in GP VI (Schmaier et al. 2009). Phosphorylation creates an ITAM motif that is recognized by the tandem SH2 domains of Syk. Association of Syk with the GP VI/FcRγ-chain complex activates Syk and leads to the phosphorylation and activation of $PLC\gamma_2$. Loss of Syk impairs collagen responses (Poole et al. 1997). $PLC\gamma_2$ hydrolyzes PIP_2, raising the cytosolic Ca^{2+} concentration and indirectly triggering Ca^{2+} influx across the platelet plasma membrane. The changes in the cytosolic Ca^{2+} concentration that occur when platelets adhere to collagen under flow can be visualized in real time (Nesbitt et al. 2003; Kulkarni et al. 2004).

4.2 Platelet Activation by ADP

ADP is stored in platelet dense granules and released upon platelet activation. It is also released from damaged cells at sites of vascular injury, serving as an autocrine and paracrine stimulus for recruiting additional platelets and stabilizing the hemostatic plug. Aggregation studies performed ex vivo show that all of the other platelet agonists are dependent to some extent on released ADP to elicit maximal platelet aggregation, although this dependence varies with the agonist and is dose-related. When added to platelets in vitro, ADP causes TxA_2 formation, protein phosphorylation, an increase in cytosolic Ca^{2+}, shape change, aggregation and secretion. It also inhibits cAMP formation. These responses are half-maximal at approximately 1 μM ADP. However, even at high concentrations, ADP is a comparatively weak activator of PLC, its utility as a platelet agonist resting more upon its ability to activate other pathways. Human and mouse platelets express two distinct GPCRs for ADP, denoted $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ receptors couple to G_q. $P2Y_{12}$ receptors couple to G_i family members other than G_z. Optimal activation of platelets by ADP requires activation of both receptors. When $P2Y_1$ is blocked or deleted, ADP is still able to inhibit cAMP formation, but its ability to cause an increase in cytosolic Ca^{2+}, shape change and aggregation is greatly impaired, as it is in platelets from mice that lack $G_{q\alpha}$ (Offermanns et al. 1997). ${P2Y_1}^{-/-}$ mice have a minimal increase in bleeding time and show some resistance to thromboembolic mortality following injection of ADP, but no predisposition to spontaneous hemorrhage. Primary responses to platelet agonists other than ADP are unaffected and when combined with serotonin, which is a weak stimulus for PLC in platelets, ADP can still cause aggregation of ${P2Y_1}^{-/-}$ platelets. Taken together, these results show that platelet $P2Y_1$ receptors are coupled to $G_{q\alpha}$ and responsible for activation of PLC. $P2Y_1$ receptors can also activate Rac and the Rac effector, p21-activated kinase (PAK), but do not appear to be coupled to G_i family members.

As had been predicted by inhibitor studies and by the phenotype of a patient lacking functional $P2Y_{12}$, platelets from ${P2Y_{12}}^{-/-}$ mice do not aggregate normally in response to ADP (Foster 2001). ${P2Y_{12}}^{-/-}$ platelets retain $P2Y_1$-associated responses, including shape change and PLC activation, but lack the ability to inhibit cAMP formation in response to ADP. The G_i family member associated with $P2Y_{12}$ appears to be primarily G_{i2}, since platelets from ${G_{i2\alpha}}^{-/-}$ mice have an impaired response to ADP (Jantzen et al. 2001; Yang et al. 2002), while those lacking $G_{i3\alpha}$ or $G_{z\alpha}$ do not (Yang et al. 2000, 2002). Conversely, expression of a $G_{i2\alpha}$ variant that is resistant to the inhibitory effects of RGS proteins produces a gain of function in mouse platelets stimulated with ADP (Signarvic et al. 2010).

4.3 Platelet Activation by Thrombin

Platelet responses to thrombin are mediated by members of the protease-activated receptor (PAR) family of GPCRs. There are four members of this family, three of which (PAR1, PAR3 and PAR4) can be activated by thrombin. PAR1 and PAR4 are

expressed on human platelets; mouse platelets express PAR3 and PAR4. Receptor activation occurs when thrombin cleaves the extended N-terminus of each of these receptors, exposing a new N-terminus that serves as a tethered ligand (Vu et al. 1991). Synthetic peptides based on the sequence of the tethered ligand domain of PAR1 and PAR4 are able to activate the receptors, mimicking at least some of the actions of thrombin. While human PAR3 has been shown to signal in response to thrombin in transfected cells, PAR3 on mouse platelets appears to primarily serve to facilitate cleavage of PAR4 rather than to generate signals on its own (Nakanishi-Matsui et al. 2000).

Thrombin is able to activate platelets at concentrations as low as 0.1 nM. Although other platelet agonists can also cause phosphoinositide hydrolysis, none appear to be as efficiently coupled to phospholipase C as thrombin. Within seconds of the addition of thrombin, the cytosolic Ca^{2+} concentration increases tenfold, triggering downstream Ca^{2+}-dependent events, including the activation of phospholipase A_2. Thrombin also activates Rho, leading to rearrangement of the actin cytoskeleton and shape change, responses that are greatly reduced or absent in mouse platelets that lack $G_{13\alpha}$. Finally, thrombin is able to inhibit adenylyl cyclase activity in human platelets, either directly (via a G_i family member) or indirectly (via released ADP) (Barr et al. 1997; Kim et al. 2000).

4.4 Platelet Activation by Epinephrine

Compared to thrombin, epinephrine is a weak activator of human platelets. Nonetheless, there are reports of human families in which a mild bleeding disorder is associated with impaired epinephrine-induced aggregation and reduced numbers of catecholamine receptors. Epinephrine responses in platelets are mediated by α_{2A}-adrenergic receptors (Kaywin et al. 1978; Newman et al. 1978; Motulsky and Insel 1982). In both mice and humans, epinephrine is able to potentiate the effects of other agonists. Potentiation is usually attributed to the ability of epinephrine to inhibit cAMP formation, but there are clearly other effects as well. Epinephrine has no detectable direct effect on phospholipase C and does not cause shape change, although it can trigger phosphoinositide hydrolysis indirectly by stimulating TxA_2 formation. These results suggest that platelet α_{2A}-adrenergic receptors are coupled to G_i family members, but not G_q or G_{12} family members. Knockout studies show that epinephrine responses in mouse platelets are abolished when $G_{z\alpha}$ expression is abolished, while loss of $G_{i2\alpha}$ or $G_{i3\alpha}$ has no effect. G_z also appears to be responsible for the ability of epinephrine to activate Rap1B (Woulfe et al. 2002; Yang et al. 2002).

4.5 Platelet Activation by TxA_2

When added to platelets in vitro, stable thromboxane analogs such as U46619 cause shape change, aggregation, secretion, phosphoinositide hydrolysis, protein

phosphorylation and an increase in cytosolic Ca^{2+}, while having little if any direct effect on cAMP formation. Similar responses are seen when platelets are incubated with exogenous arachidonate (Gerrard and Carroll 1981). Once formed, TxA_2 can diffuse across the plasma membrane and activate other platelets (Fig. 1) (FitzGerald 1991). Like secreted ADP, release of TxA_2 amplifies the initial stimulus for platelet activation and helps to recruit additional platelets. This process is limited by the brief half-life of TxA_2 in solution, helping to confine the spread of platelet activation to the original area of injury. Loss of $G_{q\alpha}$ abolishes U46619-induced IP_3 formation and changes in cytosolic Ca^{2+}, but does not prevent shape change (Offermanns et al. 1997). Loss of $G_{13\alpha}$ abolishes TxA_2-induced shape change (Moers et al. 2003). In cells other than platelets, TPα and TPβ have been shown to couple to G_i family members (Gao et al. 2001), however, in platelets the inhibitory effects of U46619 on cAMP formation appear to be mediated by secreted ADP. These observations have previously been interpreted to mean that platelet TxA_2 receptors are coupled to G_q and $G_{12/13}$, but not to G_i family members. However, the gain of function recently observed in mouse platelets carrying an RGS protein resistant $G_{i2\alpha}$ variant suggests that this is still an open issue (Signarvic et al. 2010). $TP^{-/-}$ mice have a prolonged bleeding time. Their platelets are unable to aggregate in response to TxA_2 agonists and show delayed aggregation with collagen, presumably reflecting the role of TxA_2 in platelet responses to collagen (Thomas et al. 1998). The most compelling case for the contribution of TxA_2 signaling in human platelets comes from the successful use of aspirin as an antiplatelet agent. When added to platelets in vitro, aspirin abolishes TxA_2 generation (Fig. 1). It also blocks platelet activation by arachidonate and impairs responses to thrombin and ADP. The defect in thrombin responses appears as a shift in the dose/response curve, indicating that TxA_2 generation is supportive of platelet activation by thrombin, but not essential.

5 Some of the Later Events in Platelet Activation

As platelet activation in response to injury proceeds in vivo, previously mobile platelets come into increasingly stable contact with each other, eventually with sufficient stability and proximity that molecules on the surface of one platelet can interact directly with molecules on the surface of adjacent platelets. Although in theory this can occur anywhere within a growing hemostatic plug or thrombus, it is likely to occur most readily in the thrombus core where platelets appear to be closest together. Stable cohesive contacts between platelets require engagement of $\alpha_{IIb}\beta_3$ with one of its ligands, after which inward-directed (i.e., outside-in) signaling occurs through the integrin and through other molecules that can then engage with their counterparts *in trans*. Some of these are primarily signal-generating events that affect platelet activation and thrombus stability. Others serve primarily to help form contacts between platelets and create a protected space in which soluble molecules, including agonists, can accumulate.

5.1 Outside-In Signaling by Integrins

Activated $\alpha_{IIb}\beta_3$ bound to fibrinogen, fibrin or VWF provides the dominant cohesive strength that holds platelet aggregates together. It also contributes a further impetus for sustained platelet activation by serving as a scaffold for the assembly of signaling molecules (Prevost et al. 2007; Shattil 2009; Stegner and Nieswandt 2011). The term "outside-in signaling" refers to the effects of these molecules (Shattil and Newman 2004). Some of the protein–protein interactions that involve the cytoplasmic domains of $\alpha_{IIb}\beta_3$ help regulate integrin activation; others participate in outside-in signaling and clot retraction. Proteins that are capable of binding directly to the cytoplasmic domains of $\alpha_{IIb}\beta_3$ include β_3-endonexin, CIB1, talin, kindlin, myosin, Shc and the tyrosine kinases, Src, Fyn and Syk. Some interactions require the phosphorylation of tyrosine residues Y773 and Y785 in the β3 cytoplasmic domain by Src family members. Mutation of the corresponding tyrosine residues in mice produces platelets with impaired clot retraction and a tendency to re-bleed from tail bleeding time sites (Law et al. 1999a). Fibrinogen binding to the extracellular domain of activated $\alpha_{IIb}\beta_3$ stimulates an increase in the activity of Src family kinases and Syk (Law et al. 1999a, b). Studies of platelets from mice lacking these kinases suggest that these events are required for the initiation of outside-in signaling and for full platelet spreading, irreversible aggregation and clot retraction. There is also evidence that the ITAM-containing receptor, FcγRIIa, is the link between Src family kinase and Syk activation in human platelets activated by $\alpha_{IIb}\beta_3$ (Boylan et al. 2008).

5.2 Cell Surface Ligands and Receptors

The close proximity of one platelet to another can permit the direct binding of cell surface ligands to cell surface receptors on adjacent platelets. Several examples have been identified in platelets, including members of the ephrins and semaphorin families plus their respective receptors (Eph kinases and plexins). Ephrins are cell surface molecules attached by either a GPI anchor (ephrin A family members) or a single transmembrane domain (ephrin B family members). Eph kinases have a single transmembrane domain and a cytoplasmic domain that includes the catalytic domain and protein:protein interaction motifs. Human platelets express EphA4 and EphB1 plus their ligand, ephrinB1 (Prevost et al. 2002). Forced clustering of either EphA4 or ephrinB1 results in cytoskeletal changes leading to platelet spreading, as well as to increased adhesion to fibrinogen, Rap1B activation and granule secretion (Prevost et al. 2002, 2004). EphA4 associates with $\alpha_{IIb}\beta_3$ and Eph/ephrin interactions promote phosphorylation of the β_3 cytoplasmic domain. Conversely, blockade of Eph/ephrin interactions impairs clot retraction and causes platelet disaggregation at low agonist concentrations, resulting in impaired thrombus growth. It also inhibits platelet accumulation on collagen under flow (Prevost et al. 2002, 2005).

Semaphorin 4D (Sema4D, CD100) provides another example of a cell surface ligand involved in contact-dependent signaling in platelets. Like the ephrins, semaphorins are best known for their role in the developing nervous system, but they have also been implicated in organogenesis, vascularization and immune cell regulation. Semaphorins can either be secreted, bound to the plasma membrane via a transmembrane domain or held in place with a GPI anchor. Sema4D, which has a transmembrane domain, is expressed on the surface of both mouse and human platelets and is slowly shed from the surface of activated platelets by the metalloprotease, ADAM17 (Zhu et al. 2007). Human platelets express at least two receptors for sema4D: CD72 and a member of the plexin B family (Zhu et al. 2007). Mouse platelets express the plexin, but not CD72. Sema4D$^{-/-}$ mouse platelets have a defect in their responses to collagen and convulxin in vitro, and a reduced response to vascular injury in vivo (Zhu et al. 2007). Responses to thrombin, ADP and TxA_2 mimetics are normal. The collagen defect has been mapped to a failure to maximally activate Syk downstream of the collagen receptor, GP VI. Events in the pathway upstream of Syk occur normally in sema4D$^{-/-}$ platelets (Zhu et al. 2007; Wannemacher et al. 2010). Notably, these defects are observed only when platelets come in contact with each other and can be reversed by adding soluble recombinant sema4D. Thus, the evidence suggests that sema4D provides a contact-dependent boost in collagen-signaling. The platelets which are most likely to be activated via GP VI are those in the initial monolayer that accumulates on exposed collagen.

6 Regulators of Platelet Activation

The molecular mechanisms that drive platelet activation reflect an evolutionary compromise necessitated in part by the switch from nucleated thrombocytes to the more complex system of stationary megakaryocytes and circulating platelets found in mammals. This compromise can be thought of as establishing a threshold for platelet activation. If the threshold is too high, then platelets become useless for hemostasis. If too low, then the risk for unwarranted platelet activation rises. The set point normally found in humans and other mammals is established by balancing the intracellular signaling mechanisms that drive platelet activation forward in response to injury with regulatory mechanisms that either dampen those responses or prevent their initiation in the first place.

Regulatory events that impact platelet reactivity can be viewed as being extrinsic and intrinsic. A healthy endothelial monolayer provides a physical barrier that limits platelet activation. It also produces inhibitors of platelet activation including NO, prostacyclin (PGI_2) and the surface *ecto*-ADPase, CD39, which hydrolyzes plasma ADP that would otherwise sensitize platelets to activation by other agonists. In addition to these extrinsic regulators of platelet function, a number of intrinsic regulators of platelet activation have been identified. A few of them will be

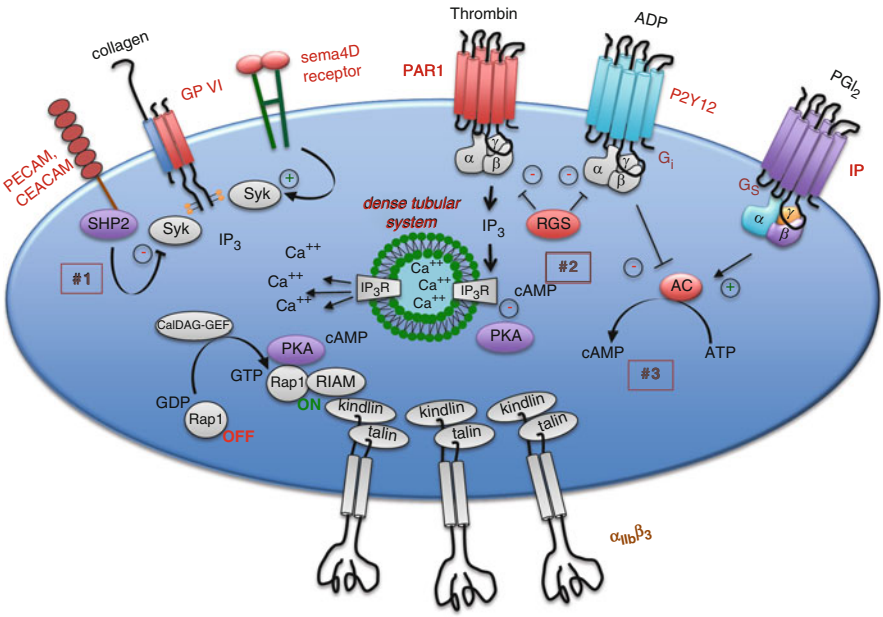

Fig. 6 Regulatory events that impact platelet activation. Although platelets are primed to respond rapidly to injury, a number of regulatory events have been described that can limit the rate and/or extent of the response. Examples shown in the figure include (1) PECAM and CEACAM, which reduce Syk activation downstream of the collagen receptor, GP VI, by recruiting the tyrosine phosphatase, SHP2, (2) RGS10 and RGS18 which shorten the duration of G protein-dependent signaling in platelets and (3) PGI_2, which stimulates cAMP formation in platelets and dampens platelet responsiveness via protein kinase A (PKA)

considered briefly here, including those that limit G protein-dependent signaling and those that impact platelet activation by collagen (Fig. 6).

6.1 Regulation of G Protein-Dependent Signaling

As already noted, most of the agonists which extend the platelet plug do so via G protein coupled receptors. The properties of these receptors make them particularly well-suited for this task. Because mechanisms exist that can limit the activation of G protein coupled receptors, platelet activation can be tightly regulated even at its earliest stages. Based largely on work in cells other than platelets, those regulatory mechanisms likely include receptor internalization, receptor phosphorylation, the binding of cytoplasmic molecules such as arrestin family members and the limits on signal duration imposed by RGS (Regulator of G protein Signaling) proteins. The role of RGS proteins in platelets is just beginning to be defined. In cells other than platelets, RGS proteins limit signaling intensity and duration by

accelerating the hydrolysis of GTP by activated G protein α subunits. At least 10 RGS proteins have been identified in platelets at the RNA level, but only RGS10 and RGS18 have been confirmed at the protein level. The evidence that RGS proteins are biologically relevant in platelets comes from studies on mice in which glycine 184 in the α subunit of G_{i2} has been replaced with serine, rendering it unable to interact with RGS proteins without impairing the ability of G_{i2} to interact with either receptors or downstream effectors. This substitution produces the predicted gain of platelet function in vitro and in vivo, even in the heterozygous state (Signarvic et al. 2010).

6.2 cAMP and Protein Kinase A

The best-known inhibitor of platelet activation is cAMP. As already noted, rising cAMP levels turn off signaling in platelets. Regulatory molecules released from endothelial cells cause $G_{s\alpha}$-mediated increases in adenylyl cyclase activity (PGI_2) and inhibit the hydrolysis of cAMP by phosphodiesterases (NO). Deletion of the genes encoding either $G_{i2\alpha}$ or $G_{z\alpha}$ causes an increase in the basal cAMP concentration in mouse platelets (Yang et al. 2002). cAMP phosphodiesterase inhibitors such as dipyridamole act as anti-platelet agents by raising cAMP levels. Conversely, loss of PGI_2 receptor (IP) expression in mice causes a decrease in basal cAMP levels, enhances responses to agonists, and predisposes mice to thrombosis in arterial injury models (Murata et al. 1997; Yang et al. 2002). Despite ample evidence that cAMP inhibits platelet activation, the mechanisms by which it does this are not fully defined. cAMP-dependent protein kinase A (PKA) is thought to be essential, but other mechanisms may be involved as well. Numerous substrates for the kinase have been described, including some G protein coupled receptors, IP_3 receptors, GP Ibβ, vasodilator-stimulated phosphoprotein (VASP) and Rap1, but it is still not clear whether a single substrate accounts for most of the effect or whether it is the accumulated effect of phosphorylation of many substrates.

6.3 Adhesion/Junction Receptors Contribute to Contact-Dependent Signaling

In addition to amplifying platelet activation, contact-dependent signaling can also help to limit thrombus growth and stability. Examples of this phenomenon include, PECAM-1, CEACAM1 and members of the CTX family of adhesion molecules. Knockouts of any of these in mice produce a gain of function, rather than a loss of function phenotype. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a type-1 transmembrane protein with six extracellular domains, the most distal of which can form homophilic interactions in *trans* (Newman and Newman 2003).

The cytoplasmic domain contains two immunoreceptor tyrosine inhibitory motifs (ITIMs) that can bind the tyrosine phosphatase, SHP-2 (Jackson et al. 1997). PECAM1-deficient platelets exhibit enhanced responses to collagen in vitro and in vivo, consistent with a model in which PECAM-1 localizes SHP-2 to its substrates, including the GP VI signaling complex, thus providing a brake and preventing excessive platelet activation and thrombus growth (Patil et al. 2001; Falati et al. 2006; Moraes et al. 2010).

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a second ITIM family member expressed on the platelet surface that can form homophilic and heterophilic interactions with other CEACAM superfamily members. In contrast to PECAM-1, the CEACAM1 ITIMs prefer SHP-1 over SHP-2, although either can become bound. The CEACAM1 knockout, like the PECAM-1 knockout produces a gain of function, showing increased platelet activation in vitro in response to collagen and increased thrombus formation in a $FeCl_3$ injury model (Wong et al. 2009). Thus, at least on the basis of the knockout results, CEACAM1 also appears to be a negative regulator of GP VI signaling.

7 Concluding Thoughts

This chapter represents our collective effort to summarize the efforts of a small, but passionate community of investigators who have worked to understand the molecular basis for platelet activation in vivo. In writing the chapter, we have focused on pathways, but have attempted to place those pathways in the context of observable events. The subject of platelet activation remains a work in progress and we have tried to incorporate perspectives that have been gained recently. We would like to close by pointing out some of the blank places on the platelet signaling map and by briefly reflecting on what might be coming next.

One of the biggest advances in the platelet field over the past 10 years has come from studies on how platelets are made. The process of proplatelet formation is described elsewhere in this volume. From the perspective of signal transduction and platelet response mechanisms, there are large unanswered questions about how a developing platelet at the tip of a proplatelet extension becomes equipped with the necessary molecular toolkit to not only drive a response to injury, but also to regulate that response to make it safe. Might some platelets be endowed with greater adhesive capabilities and be best at forming the initial monolayer on exposed collagen fibrils? Might others be particularly well suited for cohesive (platelet:platelet) interactions, either because of their complement of integrins or their density of receptors for soluble agonists such as ADP, thrombin and TxA_2? Heterogeneity among platelet populations within a single individual could easily extend to heterogeneity among individuals, perhaps contributing to risks for cardiovascular and cerebrovascular compromise (Bray 2007; Bray et al. 2007a, b; Watkins et al. 2009; Johnson et al. 2010; Kunicki and Nugent 2010; Musunuru et al. 2010). Finally, platelets contain a broad representation of megakaryocyte RNA

(Rowley et al. 2011), although not all of the proteins present in platelets are accompanied by the corresponding message (Cecchetti et al. 2011). Platelets also express the synthetic machinery needed to produce proteins (Weyrich et al. 1998; Denis et al. 2005; Schwertz et al. 2012). How do the changes that occur when platelets age in the circulation affect platelet function and how might the protein synthetic capability within the platelets help to withstand those changes or reconfigure the platelet during the hemostatic response?

A second source of recent energy in the platelet community has been the identification of new roles for platelets beyond those associated with the hemostatic response to injury. These include roles in embryonic development, innate immunity and oncogenesis. How do each of these roles draw on the molecular toolkit for platelet activation? Although the full answer to that question is not yet known, there appears to be only a partial overlap. Thus, for example, the separation of lymphatics from the vasculature depends in part on signaling pathways that are identical to those that drive platelet responses to collagen downstream of GP VI. However, instead of GP VI, the initiation of those signaling events involves a separate receptor, CLEC-2 (Hughes et al. 2010; Suzuki-Inoue et al. 2010). Similarly, platelet involvement in innate immunity appears to be driven in part by TLRs (Toll like receptors), molecules that have not appeared elsewhere in this chapter, but which are receiving increased attention for their role in platelets (Semple et al. 2011).

Knowledge Gaps

- Although many of the critical signaling pathways in platelets have been mapped, uncertainties remain about the complete mechanisms for activating $\alpha_{IIb}\beta_3$ and causing granule exocytosis.
- Gaps also remain in understanding how platelet activation is regulated so that an optimal response to local injury can be achieved without excessive accumulation of platelets.
- Platelets are generally treated as if they are all the same in each individual. However, it is not yet clear whether there are meaningful differences among platelets that optimize their ability to perform specific functions.
- Conversely, except in cases of well-defined monogenic disorders, it is not yet clear whether the differences observed in platelet aggregation among different individual donors necessarily have a definable molecular basis and a meaningful clinical impact.
- Finally, this chapter focuses on the hemostatic response to vascular injury. Uncertainty remains about the activation mechanisms that underlie pathological platelet activation. Some of them are undoubtedly the same as those employed in the hemostatic response. Others are proving not to be.

Key Messages

- Platelet activation during the response to vascular injury is a tightly coordinated process in which platelets are exposed to multiple activators and inhibitors, producing a response that is the net effect of all of these inputs.
- The most commonly encountered (and best understood) platelet agonists in vivo are thrombin, collagen, ADP, TxA_2 and epinephrine. With the exception of collagen, each of these agonists activates one or more G protein coupled receptor and signals through one or more classes of heterotrimeric G proteins. Many of these receptors have proved to prime targets for antiplatelet drugs, including the $P2Y_{12}$ and PAR1 antagonists as well as aspirin, which inhibits TxA_2 formation in platelets.
- Signal transduction during platelet activation is typically described one agonist and one pathway at a time, but it is in reality a signaling network in which different pathways can reinforce or oppose each other. Each pathway is also subject to the restraining effects of one or more inhibitors, including cAMP, RGS proteins and protein phosphatases, not all of which are fully understood.
- Once platelets are turned on, intracellular signaling that depends on a rise in cytosolic Ca^{2+} triggers a conformational change in the extracellular domain of $\alpha_{IIb}\beta_3$ (GP IIb-IIIa), exposing the fibrinogen (and fibrin) binding site on the integrin and allowing it mediate platelet aggregation.
- The development of integrin-mediated contacts between platelets makes possible a secondary wave of contact-dependent signaling that amplifies platelet accumulation and promotes formation of a stable thrombus.
- Platelet aggregation also promotes granule secretion and the release of molecules into the local surroundings that support the recruitment of additional platelets, but can also affect angiogenesis, the recruitment of leukocytes and wound healing.

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