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Current concepts of platelet activation: possibilities for therapeutic modulation of heterotypic *vs.* homotypic aggregation

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Thrombogenic and inflammatory activity are two distinct aspects of platelet biology, which are sustained by the ability of activated platelets to interact with each other (homotypic aggregation) and to adhere to circulating leucocytes (heterotypic aggregation). These two events are regulated by distinct biomolecular mechanisms that are selectively activated in different pathophysiological settings. They can occur simultaneously, for example, as part of a pro-thrombotic/pro-inflammatory response induced by vascular damage, or independently, as in certain clinical conditions in which abnormal heterotypic aggregation has been observed in the absence of intravascular thrombosis. Current antiplatelet drugs have been developed to target specific molecular signalling pathways mainly implicated in thrombus formation, and their ever increasing clinical use has resulted in clear benefits in the treatment and prevention of arterial thrombotic events. However, the efficacy of currently available antiplatelet drugs remains suboptimal, most likely because their therapeutic action is limited to only few of the signalling pathways involved in platelet homotypic aggregation. In this context, modulation of heterotypic aggregation, which is believed to contribute importantly to acute thrombotic events, as well to the pathophysiology of atherosclerosis itself, may offer benefits over and above the classical antiplatelet approach. This review will focus on the distinct biomolecular pathways that, following platelet activation, underlie homotypic and heterotypic aggregation, aiming potentially to identify novel therapeutic targets.

Introduction

The classical model of platelet activation describes a series of phenotypic and morphological changes of resting platelets such that they acquire adhesiveness to the vascular wall in response to their exposure to pro-thrombotic molecules [e.g. collagen, von Willebrand factor (vWF)] [1–3], forming platelet-to-platelet aggregates (homotypic aggregation) through interlinking by soluble adhesive proteins (fibrinogen) [4] and releasing a number of mediators that stabilize the initial aggregate and amplify thrombus formation [5]. The widespread view that vessel injury is the sole determinant of platelet activation has been largely abandoned in light of the evidence that a variety of pathophysiological stimuli, including pro-inflammatory cytokines and infective agents (recently reviewed by Semple & Freedman [6]) as well as shear stress [7], can activate platelets with no detectable vessel damage. Evidence has also accumulated that, in these and other diverse pathophysiological settings, platelets exhibit differential biological responses because of activation of distinct biomolecular pathways. Importantly, pro-inflammatory activity mediated by interaction with circulating leucocytes (heterotypic aggregation) and release of inflammatory mediators constitutes an important aspect of platelet biology and provides strong pathophysiological links between inflammation and thrombosis [6, 8]. As a consequence, platelet activation is not limited to the occurrence of a local thrombotic event triggered by vascular damage and mainly sustained by homotypic aggregation. On the contrary, it is often part of a systemic inflammatory response that can develop independently of, or in addition to, local injury-related factors. Indeed, formation of heterotypic aggregates in the peripheral circulation has been observed not only during acute thrombotic events [9–12], but also in those clinical conditions associated with high blood thrombogenicity in the absence of intravascular thrombosis, such as auto-immune disorders [13], haematological disease [14, 15] and in subjects with cardiovascular risk factors [16–18]. Given this, as well as the ongoing discovery

Table 1
Comparison between homotypic and heterotypic aggregation

	Homotypic aggregation	Heterotypic aggregation
Localization	Confined to the vascular wall	Detectable in peripheral blood
Platelet activation required	Not under high shear rate	Yes
Mechanism of stabilization	Multiple ligand-receptor interactions with thrombogenic surface (demonstrated)	Multiple ligand-receptor interactions with leucocyte membrane (hypothesized)
Main effector molecule(s)	Varies depending on haemodynamic condition and lesion components	P-selectin, P-selectin glycoprotein ligand-1
Platelet release reaction	Required for irreversible stabilization, mainly mediated by dense granule contents	Required for initiation, mediated by alpha granule contents

of ever more biomolecular mechanisms underlying the platelet response to different agonists, it is likely that the signalling pathways that regulate thrombus formation and platelet–leucocyte interaction may operate either together or independently. In keeping with this, some antiplatelet drugs that effectively reduce platelet–platelet aggregation, such as aspirin, may have very little or no effect on heterotypic aggregation [19].

The increasing interest in pharmacological inhibition of heterotypic complex formation stems from the concept that platelet–leucocyte adhesion is not a simple epiphenomenon associated with thrombus development but represents a pro-thrombotic as well as a pro-atherogenic mechanism in itself. Indeed, interaction of activated platelets with white cells amplifies platelet activation and favours homotypic aggregation at sites of vascular injury [20], as well as exerting a damaging action on the vascular wall thereby predisposing to atherosclerotic disease [21, 22]. These observations raise the important question of whether inhibition of heterotypic aggregation might offer additional therapeutic benefit over and above the classical antithrombotic strategy, where the principal target and effect is platelet–platelet aggregation and thrombus formation.

We will here review the biomolecular mechanisms that are differentially implicated in homotypic and heterotypic aggregation, with a view to identifying, on the basis of recent data, potential therapeutic targets to prevent both the pro-thrombotic and pro-inflammatory effects of platelet activation. Table 1 compares some important features of homotypic and heterotypic aggregation.

Homotypic aggregation

Homotypic aggregation occurs at the site of a vascular lesion such as a ruptured atherosclerotic plaque or a traumatic injury, where exposed pro-thrombotic molecules provide an adhesive surface for recruitment of circulating platelets, followed by their activation and thrombotic plug formation [1–5]. *In vivo* and *in vitro* observations suggest that this phenomenon is a complex and dynamic multistep process [20, 23, 24]. The initial phase of adhesion of platelets to the vascular wall as well as to each other (primary reversible aggregation) is followed by a second phase of stabilization and growth of the initial platelet plug (secondary irreversible aggregation). Platelet activation has long been assumed to have a dual role in this process, as an initiating factor in platelet arrest and as an essential mediator of the transition from reversible to irreversible aggregation [1, 25, 26]. Technical advances in intravital microscopy and real-time perfusion studies have demonstrated that primary aggregation can also occur without the need for platelet activation under conditions of elevated shear stress [24, 27]. However, when non-activated platelets adhere to the vessel wall they only form transient micro-aggregates that, in the absence of activation-dependent release and generation of soluble agonists [principally adenosine diphosphate (ADP), thrombin and thromboxane A_2 (TxA_2)], disaggregate with translocation of platelets in the direction of flow [24]. Central to homotypic aggregation is therefore the concept that platelets become activated in response to interaction with thrombogenic surfaces, and multiple ligand-receptor interactions are required to stabilize and amplify their adhesion and aggregation.

Biomolecular mechanisms of platelet activation leading to homotypic aggregation

Fibrinogen, vWF and collagen are able to initiate primary aggregation through the engagement of specific platelet integrins, namely glycoprotein (GP) IIb/IIIa (also designated $\alpha_{2b}ß_3$ integrin), GPIb and GPVI, respectively [25, 28, 29]. At low shear rate (<1000 s^{-1}), the interaction between GPIIb/IIIa and fibrinogen has been demonstrated to constitute the predominant biomolecular event [1, 25, 30]. However, as GPIIb/IIIa is expressed in a low affinity state on the plasmalemma of quiescent platelets, initial stimulation of platelets by one or more soluble agonists in the vicinity of the lesion (e.g. ADP released from endothelial cells or

thrombin locally produced) is required in order to activate downstream signalling pathways (inside-out signalling) that ultimately result in platelet shape change and activation of GPIIb/IIIa [4]. When the shear rate rises within the range 1000–10 000 s^{-1}, platelet activation is not required to induce primary aggregation, as the synergistic action of GPIIb/IIIa and GPIb suffice in promoting tethering and transient aggregation of discoid-shaped quiescent platelets to the vascular wall. Nevertheless, the ensuing activation of platelets induced by integrin engagement leads to release of soluble agonists, mainly ADP, which is essential in stabilizing the initial aggregate [24]. At high shear rates (>10 000 s^{-1}), Ruggeri *et al*. have shown both *in vitro* and *in vivo* that a thrombus can form efficiently through a mechanism independent of platelet activation, that is solely mediated by interaction between vWF and GPIb giving rise to stable local adhesion of platelets to a thrombogenic surface and homotypic aggregation [27].

The *in vivo* role of these ligand/receptor interactions has been evaluated in animal models selectively lacking one or more of the molecules involved in these pathways. In studies using vWF–/– mice, platelet accumulation and thrombus growth were markedly delayed but not absent in a model of ferric chloride-induced thrombosis [31], and the thrombogenic activity of platelets in laser-induced vessel wall injury was in fact comparable with that observed in wild-type mice [32], suggesting that platelet thrombus formation can occur in the absence of vWF. Fibrinogen/vWF knockout mice exhibit preserved platelet-to-platelet interaction [31], via a mechanism primarily triggered by thrombin and sustained by soluble agonist release (ADP) and different integrin signalling cascades [32, 33]. Activation of the coagulation cascade at the site of vessel injury, with consequent generation of thrombin through the tissue factor (TF) pathway, has been proposed as a major contributor to the thrombogenic component of atherothrombotic disease [34, 35]. However, recent evaluation of the dynamics of thrombus formation on atherosclerotic plaques has shown that TF has a predominant role only in the amplification phase of platelet aggregation, while the first key event of platelet arrest and aggregation is crucially regulated by engagement of the collagen receptor GPVI [36]. Indeed GPVI blockade, but not plaque TF suppression, significantly inhibits thrombus development. In similar experiments performed by Penz *et al*. [37], GPIb was found to be a crucial effector in plaque-induced thrombus, a finding in agreement with the work of Ruggeri *et al*. showing the importance of the vWF/GPIb axis in thrombus formation under conditions of high shear, and further confirmed in studies of interleukin 4-receptor/GPIb transgenic mice [38], in which lack of activity of GPIb gives rise to a severe bleeding phenotype. However, compared with the relatively mild effect on thrombogenic response to vascular injury observed in vWF-knockout mice, these results are strongly suggestive of an additional thrombogenic mechanism sustained by GPIb that may interact with ligands other than vWF.

Taken together, these findings imply that, independent of the initial thrombogenic stimulus and blood flow conditions, platelet-to-platelet interaction only results in irreversible aggregation once stable adhesion to the vascular wall, mediated by multiple receptor-ligand binding events, is established.

Heterotypic aggregation

The ability of platelets to interact with leucocytes was established in the late 19th century [39], from intravital microscopic studies of Bizzozero who first discovered platelets and reported the description of both homotypic and heterotypic aggregation at the site of vascular injury: 'Blood platelets, swept along by the blood stream, are held up at the damaged spot as soon as they arrive at it. At first, one sees only two to four to six (platelets); very soon the number climbs to hundreds. Usually some white blood cells are held up amongst them.' (Taken from a review by Brewer [40]).

More than a century of research in the field has provided multiple lines of evidence supporting the concept that, by contrast with homotypic aggregation, which is confined to the vascular wall, heterotypic complex formation occurs in circulating blood. The first detection of leucocyte–platelet aggregation in the human circulation was reported in subjects with coronary atherosclerosis [41], in whom heterotypic aggregates were identified within the coronary circulation, in close proximity to atherosclerotic lesions. The same study reported a higher level of heterotypic complexes in the vicinity of plaques with thrombotic complications as compared with uncomplicated ones. In further clinical studies, heterotypic complexes were observed in the systemic circulation of patients with coronary atherosclerosis [9–11], their levels being increased during acute thrombotic events. These findings suggest that circulating heterotypic aggregates form in parallel with thrombi, as a consequence of local platelet activation sustained by vascular damage. However, the presence of atherosclerotic lesions may not be mandatory for heterotypic aggregation, as raised levels of such aggregates have been described also in healthy subjects with cardiovascular risk factors [16–18], although such subjects may well have subclinical atherosclerosis, as well as in patients with other inflammatory conditions (reviewed by von Hundelshausen & Weber [42]). Adhesion between platelets and leucocytes is stable, does not require adhesion to pro-thrombotic surfaces, and is a systemic phenomenon whose extent depends on the degree of platelet activation. Early *in vitro* experiments performed on whole blood showed that platelet activation by a variety of agonists results initially in homotypic interaction mediated by

fibrinogen-GPIIb/IIIa interaction, which is followed, in the absence of an adhesive vascular surface, by platelet disaggregation and subsequent platelet adhesion to leucocytes, with monocytes having a competitive advantage over other white cells in binding activated platelets [43]. Inhibition of fibrinogen binding to GPIIb/IIIa is even able to enhance the interaction of activated platelets with leucocytes, and the heterotypic complexes so formed, that are mainly composed of monocyte–platelet aggregates, display a strong pro-inflammatory phenotype mediated by surface-expressed molecules such as CD40L and P-selectin, along with pro-thrombotic activity sustained by their production of TF [44].

Biomolecular mechanisms of platelet activation leading to heterotypic aggregation

The pioneering work of Jungi *et al*. in 1986 [45] demonstrated that platelets acquire adhesiveness to leucocytes when stimulated with thrombin in a concentration-dependent manner, while non-activated platelets show little tendency to associate with white cells, any such association being explained by low-grade spontaneous activation of platelets undergoing handling *in vitro*. A number of other researchers confirmed these results [43, 46, 47], and it is now well established that platelet activation, independent of the initial stimulus, is required to trigger platelet-leucocyte interaction. As part of the underlying molecular mechanism, expression of the adhesion molecule P-selectin on the plasmalemma of activated platelets appears to be crucially involved [47, 48]. P-selectin, also designated CD62P and previously referred to as GMP-14O or PADGEM protein, is a transmembrane molecule stored in the alpha-granules of platelets. Upon stimulation, the granules fuse with the plasma membrane and P-selectin thus translocates from the cytosolic compartment to the extracellular surface, where it acts as a receptor for circulating white cells [48]. Its specific ligand is known as P-selectin glycoprotein ligand (PSGL)-1, and this is constitutively expressed on the plasmalemma of leucocytes [49]. Within the leucocyte population, the expression of PSGL-1 on the cellular surface of monocytes seems to be highest [50], and this could in part explain their elevated binding affinity to platelets over other white cells. Indeed, an *in vivo* study performed in apoE–/– mice [21] demonstrated that intravenous injection of activated platelets leads to sequestration of leucocytes within a few (approximately 5) minutes, persisting up to 80 min for polymorphonuclear cells (PMN) and 180 min for monocytes. No such effect was reported for lymphocytes, confirming once more the differential binding activity of activated platelets to different leucocyte sub-populations, as observed in early *in vitro* studies [43, 44]. A similar study in baboons showed similar results [9]: after injection of activated platelets, formation of circulating aggregates with monocytes and PMN occurred within 1 min, and the *in vivo* half-lives of circulating monocyte–platelet and PMN–platelet complexes were, respectively, 30 and 5 min; no heterotypic aggregates were detectable after 2 h.

The preferential binding of platelets to monocytes may also be ascribed to an additional role played by other ligands specifically present on the plasmalemma of monocytes, which may follow on from P-selectin/PSGL-1 interaction. Indeed, PSGL-1 is not only an adhesion but also a signalling molecule. PSGL-1 binding induces production of superoxide anion radicals from monocytes [51], and tyrosine phosphorylation of various cytoplasmic proteins, including pp125 focal adhesion kinase, ERK, Syk, Src kinase and paxillin [52–55]. Binding of PSGL-1 to P-selectin leads to integrin activation on monocytes, resulting in further stabilization of cellular adhesion in part mediated by additional bridging molecules. Further interactions are through CD40L/CD40, TREM-1 ligand/TREM-1 (which may also promote integrin expression) and CD36/CD36 via thrombospondin [56]. However, P-selectin/PSGL-1 linking clearly has a crucial role in heterotypic aggregation, as blocking monoclonal antibody to P-selectin [45] or PSGL-1 [16] completely abolishes the formation of leucocyte–platelet aggregates.

Platelet release reaction

Release of platelet granule contents represents a common biomolecular event in both homotypic and heterotypic aggregation (Table 1). In the former, it contributes to the amplification and stabilization of initial reversible aggregates, mainly through the release of platelet agonists. In the latter, it sustains the receptor-ligand interactions between platelets and white cells by promoting the expression of P-selectin. Platelet agonists and adhesion molecules are segregated into specific intracellular compartments [57, 58] and, for many decades, their release was considered to be directed by a structural re-organization of the cytoskeleton [59–61]. This conventional view of the platelet release reaction has been recently extended to include a more composite mechanism of fusion between the granule membrane and the plasma membrane, subtly regulated by N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family proteins, chaperon and the composition of the lipid membrane (reviewed by Reed *et al*. [62] and Flaumenhaft [63]). The activity of this regulatory apparatus seems to be modulated by agonist-specific effector molecules, to ensure selectivity in dense and alpha granule content release as appropriate.

Platelet granules and selective regulation of release reaction

Immunocytochemical and electron microscopy studies over the years have given rise to the distinction between three main types of storage organelles in platelets, known

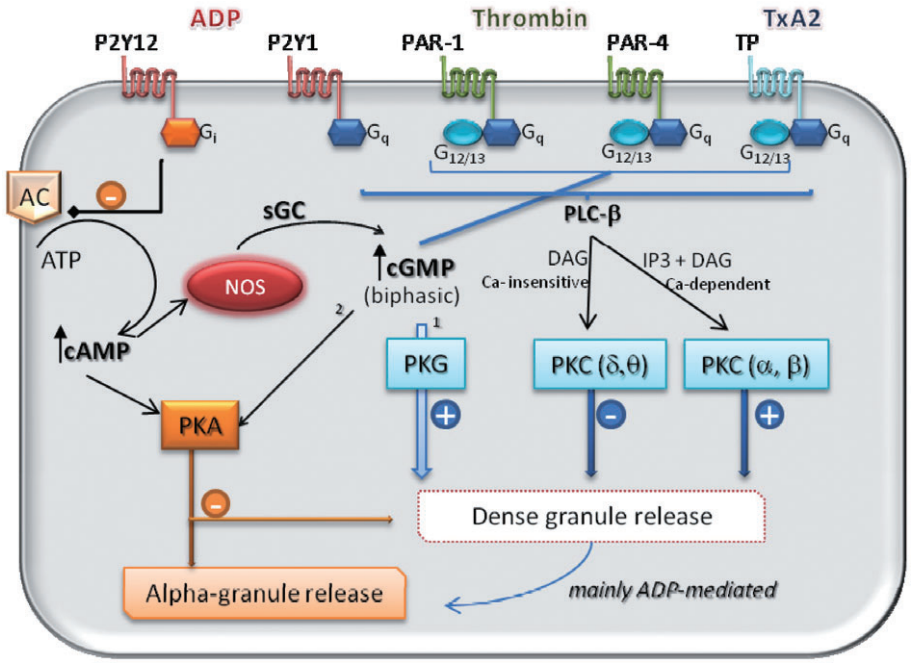

Figure 1

Model of agonist-selective modulation of alpha and dense granule release in human platelets

as dense, alpha and lysosomal granules. Their content can be classified into three main groups on the basis of their principal function: platelet agonists [ADP, serotonin, epinephrine, 5-hydroxytryptamine (5-HT) and calcium], contained in the dense granules, adhesion molecules [P-selectin, fibrinogen, vWF, platelet factor 4 (PF4)], stored in alpha-granules and lysosomal enzymes (cathepsin, hexosaminidase) within the lysosomes [64]. Ultrastructural and morphological studies have revealed the intracellular organization of the different organelles, which distribute along an intricate network of membrane invaginations forming an open canalicular system (OCS) [64]. The process of actin polymerization in activated platelets has been generally considered to be the major determinant in organelle trafficking, because of the generation of contractile force with consequent expulsion of granules [59–65]. More recent studies have revealed that platelet shape changes, as sustained by actin re-organization following platelet stimulation, are not essential for the platelet release reaction [66–68]. Indeed, actin disruption has shown either inhibitory or stimulatory effects on platelet degranulation in different studies [66–70]. The discrepancy in results between these studies may be in part ascribed to the evidence, recently provided by Flaumenhaft *et al*. [71], that the cytoskeleton differentially regulates dense and alpha granule exocytosis; specifically, low grade actin disruption stimulates agonist-induced alpha granule release with only minimal effect on dense granule secretion, while high levels of actin disruption inhibit alpha granule release but promote dense granule secretion. The molecular mechanisms underlying the platelet release reaction are still poorly understood, but increasing evidence suggests the existence of distinct regulatory pathways for alpha and dense granule secretion (Figure 1). It has recently been reported that Munc13 proteins, which act in concert with the SNARE complex in mediating fusion between the granule/vesicle membrane and the plasma/target membrane [72], provide a single and common mechanism for the release reaction of all the different types of platelet granule. However, the residual secretion of alpha granule contents observed in response to thrombin in Munc13-4 knockout mice, in which dense granule release is totally abolished, suggests that secretion of alpha granules is less dependent on Munc13-4 activity than that of dense granules [72]. The scenario is complicated by recent observations that alpha organelles are composed of different subtypes of vesicle containing different adhesion molecules, which are released independently of each other [73–75].

Given the fact that soluble molecules stored in dense organelles are predominantly involved in homotypic aggregation and thrombus formation, while P-selectin in alpha granules is mainly implicated in heterotypic complex formation, it is conceivable that, under different pathophysiological circumstances, different types of platelet release reaction occur. The distinct biomolecular pathways involved may differentially favour thrombus formation and/or platelet interaction with white cells.

Pharmacological modulation of homotypic and heterotypic aggregation

Aspirin and thienopyridine derivatives, including clopidogrel and prasugrel, are the most widely used antiplatelet drugs in clinical practice, especially in the chronic management of atherosclerotic disease. Clinical studies report a differential ability of these two classes of agent to modulate homotypic and heterotypic aggregation. In the following, we will examine the molecular mechanisms underlying their therapeutic action, in parallel with the molecular pathways activated in response to different platelet agonists, with a view to clarifying how the selective modulation of these different signalling pathways exerts differential effects on these two events which may allow identification of potential new therapeutic targets.

Aspirin

The antiplatelet effect of aspirin is mediated by the irreversible inhibition of thromboxane (Tx)A_2 synthesis in platelets, because of acetylation of a serine hydroxyl group at position 529 in cyclo-oxygenase (COX)-1. This steric modification of the enzyme inhibits the access of arachidonic acid, the precursor of TxA_2, to the active site of the enzyme. Blockade of COX-1 in platelets, as obtained either following *in vivo* treatment or *in vitro* incubation of platelets with aspirin, usually gives rise to effective inhibition of aggregation in response to a number of platelet agonists, although there is evidence of reduced aspirin response in some subjects, giving rise to the concept of aspirin resistance [76]. The antiplatelet effect of aspirin is mainly ascribed to negative modulation of dense granule release. Indeed, an early study reported that alpha granule exocytosis, as detected by P-selectin expression, vWF and beta-thromboglobulin secretion, was not at all influenced by COX-1 blockade in platelets stimulated with either ADP or thrombin [77]. These data imply that TxA_2 signalling is primarily involved in dense granule but not alpha granule secretion.

In accordance with this, Li *et al*. reported no effect in healthy subjects of aspirin either on platelet P-selectin expression or on platelet–leucocyte aggregation, after stimulation with ADP, thrombin or platelet activating factor [78]. Similarly, Klinkhardt *et al*. found that aspirin did not affect the formation of leucocyte–platelet complexes in the circulation [19], and Moshfegh *et al*. demonstrated a reduced effect of aspirin when compared with other antiplatelet drugs in reducing platelet P-selectin expression [79]. As heterotypic aggregates are believed to promote atherosclerosis progression through sustaining a pro-inflammatory state, this relative lack of effect of aspirin on heterotypic aggregation may explain, at least in part, the lack of any clear effect on atherosclerosis progression as well as the poorer results on clinical outcome as compared with newer antiplatelet agents such as the thienopyridines, as discussed further below [80].

Thienopyridines

Ticlopidine, clopidogrel and prasugrel are selective, irreversible noncompetitive inhibitors of the $P2Y_{12}$ receptor. All are prodrugs that require oxidation by hepatic cytochrome P450 to be converted into active metabolites [81]. Their effect on platelet aggregation has been investigated in many clinical trials, whose main end point was the reduction of fatal or non-fatal cardiovascular (CV) events [82–86]. Clear benefit from $P2Y_{12}$ receptor blockade has been consistently found in the secondary prevention of CV disease. Compared with ticlopidine and clopidogrel, prasugrel provides faster and more uniform inhibition of $P2Y_{12}$-dependent platelet activation [87], resulting in reportedly more effective reduction of major thrombotic events. However, the higher platelet inhibition achieved with prasugrel also gives rise to increased incidence of bleeding events compared with clopidogrel [88]. Reversible $P2Y_{12}$ antagonists, such as cangrelor and ticagrelor, which belong to the cyclopentyl-triazolo-pyrimidine chemical class, have been recently developed [89] and offer a clinically useful alternative to thienopyridines. Unlike cangrelor, ticagrelor has shown superiority to clopidogrel in reducing CV events, as well as the advantage of shorter time of offset if surgery is contemplated (24–72 h) compared with thienopyridines which take at least 5 days to wear off [90].

Beyond a direct effect on homotypic aggregation, antagonism of $P2Y_{12}$ receptor has demonstrated efficacy in reducing CD40L and P-selectin expression, as well as platelet–leucocyte aggregate formation [91]. In accordance with these findings, thienopyridines, but not aspirin, have been shown to reduce platelet P-selectin expression, which can be explained by their ability to inhibit ADP-induced alpha-granule release [19, 91]. Prasugrel has shown greater efficacy than clopidogrel in modulating heterotypic aggregation and P-selectin expression, in ADP-stimulated platelets obtained from subjects with atherosclerosis treated with the two different thienopyridines [92]. Thus, $P2Y_{12}$ blockade may modulate vascular inflammation in addition to platelet thrombosis [93].

G_q protein-coupled receptors and the platelet release reaction

TxA_2, ADP and thrombin all induce platelet activation through G-protein coupled receptors (GPCR). The TxA_2 receptor (TP) couples to the G_q and $G_{12/13}$ subtypes, as do the thrombin receptors protease activated receptor (PAR)-1 and PAR-4. $G_{12/13}$-dependent signalling pathways positively feed back on the G_q signal-transduction cascade. ADP binds two main purinoceptors, namely $P2Y_1$ and $P2Y_{12}$, which, respectively, couple to G_q and G_i [94].

The main effector of G_q is phospholipase C (PLC), mainly the PLC-β isoform, which via hydrolysis of membrane

phospholipids and consequent generation of two second messengers, inositol trisphosphate (IP3) and diacylglycerol (DAG), leads to the activation of protein kinase C (PKC) [5]. PKC comprises three groups of isoforms: the classical subgroup (α, β, γ), whose activation is calcium-dependent and mediated by the synergistic action of IP3 and DAG, the calcium-insensitive isoforms (δ, η, θ, ε), regulated by DAG and the IP3-sensitive atypical isoforms [95, 96]. The enzymatic activity of PKC has been demonstrated to have important implications in the regulation of the platelet release reaction [97, 98], as a consequence of its ability to phosphorylate and thereby activate the SNARE machinery [99]. Ablation of PKC-α in murine models gives rise to defective responsiveness of platelets to *in vitro* stimulation with thrombin, because of compromised release of dense granule contents. Indeed, platelets lacking expression of PKC-α show a decrease in ATP secretion compared with wild-type platelets, and the addition of exogenous ADP restores agonist-dependent aggregation [100]. The expression of P-selectin, an indicator of alpha-granule release, is also impaired in response to thrombin when PKC-α is not present. However, in these experiments, the ability of ADP to restore translocation of P-selectin to the platelet plasmalemma was not evaluated, so it is not known whether ADP also restores alpha-granule secretion in this model.

In separate *in vitro* experiments, the specific role of the PKC-δ isoform in granule content release was evaluated. Murugapan *et al*. [101] demonstrated that TxA_2 and thrombin induce PKC-δ phosporylation, and that this effect is accompanied by dense granule exocytosis which is abolished by selective inhibition of PKC-δ activity. However, in collagen-stimulated platelets, PKC-δ was found to exert an inhibitory effect on platelet activation, implying that different stimuli may activate distinct transduction pathways. Similarly, ADP was not found to exert any modulatory action on PKC-δ. An individual comparison between the roles of different PKC subtypes has been recently reported by Gilio *et al*. [102], who used pharmacological approaches in human platelets as well as specific knockout mice to study platelet function under physiological flow conditions and in response to collagen. They demonstrated that activation of conventional Ca^{2+}-dependent PKC isoforms stimulates dense granule release and platelet aggregation, while activation of Ca^{2+}-insensitive isoforms inhibits these events (Figure 1). Moreover, although PKC-δ was found to regulate negatively dense granule release, no effect on alpha granule secretion was observed. However, *in vivo* studies indicate a co-adjuvant rather than essential role of PKC in thrombus formation. In PKC-α-deficient mice, no alteration of bleeding time or thrombus formation compared with wild-type mice is seen [100], despite a decrease in the volume of thrombus compared with wild-type controls in both PKC-α and PKC-β knockout mice secondary to compromised platelet-to-platelet adhesion [102].

The ability of ADP to correct the defect in platelet function in the presence of PKC deficiency demonstrates that its action on platelets is mediated by alternative signalling pathways. Its ability to activate G_i as well as G_q protein-dependent signalling seems to represent a crucial mechanism in ADP-mediated platelet activation, as specific inhibition of the former pathway has been demonstrated to compromise its co-adjuvant role in thrombin- or TxA_2-mediated platelet stimulation [103, 104].

G_i protein-coupled receptors and adenylyl cyclase

The interaction of ADP with the $P2Y_{12}$ receptor activates G_i-mediated signalling that, via negative modulation of adenylyl cyclase (AC) enzymatic activity, reduces intracellular concentrations of cyclic AMP (cAMP) [105]. AC represents an endogenous inhibitory mechanism of platelet activation, whose action is mediated by cAMP-dependent activation of protein kinase A (PKA). PKA-mediated inhibition of platelet activation involves interference with calcium release [106], phosphorylation of cytoskeletal proteins such actin binding protein (ABP) and myosin light chain kinase (MLCK) [107], as well as of the focal adhesion mediator vasodilator-stimulated phosphoprotein (VASP) [108]. The inhibition of AC, as caused by $P2Y_{12}$ activation, therefore results in amplification of activator pathways triggered by different agonists. Indeed, $P2Y_{12}$ stimulation potentiates the effect of thrombin and TxA_2 on platelet aggregation as well as increasing P-selectin expression [103, 104]. Evidence for the essential role of G_i-dependent pathways in regulating the platelet release reaction has been provided by Li *et al*. [109], who studied signal-transduction regulated by TxA_2 and thrombin in an animal model lacking expression of protein kinase G (PKG). They demonstrated that these two soluble agonists activate PKG, a cyclic GMP (cGMP)-dependent tyrosine kinase enzyme, through their specific G_q-protein coupled receptors. PKG activation plays an important role in dense granule release from platelets. Nevertheless, the impaired platelet response to thrombin and TxA_2 observed in PKG knockout compared with wild-type animals was counteracted by addition of exogenous ADP, an effect abolished by selective G_i inhibition. The same findings were obtained in human platelets, in which blockade of G_i signalling nullified the potentiating effect of ADP on thrombin and TxA_2 stimulation of platelets. Of note, recent work from Iyu *et al*. [110] has demonstrated that G_i blockade, as occurs in response to $P2Y_{12}$ antagonists, converts the stimulatory activity of ADP on platelet activation into an inhibitory effect mediated by ADP-derived adenosine.

Other antagonists/agonists and pathways modulating adenylyl cyclase activity

Adenosine is a purine nucleoside that is formed from the sequential dephosphorylation of ATP. Adenosine modulates platelet function through the engagement of its specific receptors A_{2A} and A_{2B}, that couple to stimulatory

G_s-proteins thereby activating AC. The resulting increase in cAMP gives rise to inhibition of platelet aggregation [110].

Epinephrine is contained in platelet dense granules and is released upon platelet activation [111, 112]. It acts on α_{2A}-adrenergic receptors, coupled to G_z-proteins which belong to the G_i family [113, 114]. Epinephrine is considered a weak platelet agonist, as alone it does not sustain platelet aggregation in the absence of other agonists [94]; rather, it sensitizes platelets to the action of other agonists [115], and is able to induce P-selectin expression on platelets as well as to sustain platelet–leucocyte aggregation [116].

Prostaglandin E_2 (PGE_2) modulates platelet function via its binding to EP3 receptors, which are coupled to G_i protein [94] and EP4 receptors, which feed back on G_s-protein [117]. As with epinephrine, PGE_2 is not an aggregating agent *per se* at low concentrations, but potentiates the effect of other agonists, as demonstrated in both *in vitro* experiments and *in vivo* animal models [118, 119]. The pro-aggregatory effect of PGE_2 has been recently demonstrated to be secondary to cAMP reduction, as a consequence of EP3 receptor activation. On the other hand, PGE_2 binding to EP4 induces an opposite inhibitory effect on platelet function, mediated by increased cAMP [117]. Similarly opposing effects on platelet activity have been described for prostaglandin E_1 (PGE_1) which, via binding to EP3 receptors, reduces cAMP thereby promoting platelet activation, while on the other hand, by interacting with IP receptors (as does prostacyclin), inhibiting platelet function [120].

All the chemokine receptors identified on the platelet plasmalemma, including CCR1, CCR2, CCR3, CCR4, CXCR4 and CX3CR1, are coupled to G_i. They all are considered weak platelet activators, but their role appears to be predominantly in the inflammatory response that accompanies atherosclerotic vascular disease and thrombosis [94].

Overall, the evidence suggests that negative modulation in platelets of the basal activity of AC, resulting in decreased cAMP, favours their aggregation, and even in the absence of effective homotypic aggregation, this mechanism suffices to induce platelet P-selectin expression and consequent heterotypic aggregation.

Pharmacological modulation of adenylyl cyclase: effect on heterotypic aggregation

Stimulation of AC with consequent cAMP elevation prevents platelet activation and adhesion. This effect has been observed in response to stimulation of G_s-coupled β-adrenoceptors in platelets that, via cAMP-dependent activation of nitric oxide (NO) synthase (NOS) type 3, primarily reduce platelet heterotypic adhesion with minimal, or no effect, on homotypic aggregation [121]. Similarly, in a study of healthy subjects, *ex vivo* treatment of platelets with exogenous NO was found to decrease their aggregation with monocytes [16]. In line with these results, Morell *et al*. [122] have shown that NO inhibits exocytosis of dense and alpha-granules, but the effect on the latter is more pronounced than on the former, as it is evident at concentrations as low as 10 nM compared with 1 μM required to modulate significantly dense granule release. Conversely, decreased NOS activity enhances monocyte–platelet complex formation [16, 123]. The effect of NO on platelet activation is mediated through soluble guanylyl cyclase (sGC) and consequent increase in cGMP. It has been reported by one group that cGMP plays a biphasic role on the platelet release reaction, with an early stimulatory action mediated through PKG and a late inhibitory effect occurring at higher cGMP concentrations and in a PKA-dependent manner [124, 125], although this is controversial.

Cilastozol, which is a phosphodiesterase (PDE) 3 inhibitor, can increase platelet cAMP [126], as well as efficiently suppress P-selectin expression and platelet–leucocyte aggregate formation induced by a variety of agonists [126, 127]. Addition of cilastozol to standard dual antiplatelet therapy (aspirin plus clopidogrel) in patients undergoing coronary artery stent implantation has been reported to result in increased inhibition of platelet aggregation and P-selectin expression, in *ex vivo* functional assays [128].

Pharmacological modulation of the serotoninergic system can modify platelet activation status and heterotypic complex formation. Indeed, selective serotonin re-uptake inhibitors, commonly used for the treatment of depression, have been shown to inhibit platelet–leucocyte aggregation [129, 130], most likely as a consequence of their demonstrated ability to activate AC in platelets [131]. Similar findings have been reported in rodents treated with herbal medicines such as magnolol and honokiol from *Magnolia officinalis* [132], used for treatment of depression in traditional Chinese medicine, as well as curcumin from *Curcuma longa* [133], used by alternative practitioners for improving cognitive function and for its purported anti-inflammatory and antitumour effects, all of which enhance platelet AC activity and thus intracellular cAMP.

From all of these data, G_i-dependent signalling appears to be crucially implicated in alpha granule release and consequent heterotypic aggregation, independently of other biomolecular events activated during platelet adhesion to the vascular wall and thrombus formation. At the same time, it exerts a modulatory effect on platelet activation and homotypic aggregation induced by agonists whose direct action is G_q-mediated.

Table 2 summarizes the effects of different agents that modulate intraplatelet cAMP on homotypic and heterotypic aggregation.

Novel therapeutic approaches to modulation of homotypic and heterotypic aggregation

The development of new drugs which target the above described molecular mechanisms underlying adhesion

Table 2

Effects of platelet agonists/inhibitors on intraplatelet cAMP and consequent effects on homotypic *vs.* heterotypic aggregation

	cAMP modulation	Effect on homotypic aggregation	Effect on heterotypic aggregation
ADP	↓	Stimulatory, requires activation of both P2Y1 and P2Y12	Stimulatory, mainly sustained by P2Y12
Adenosine	↑	Inhibitory	Inhibitory
Epinephrine	↑	Very little	Stimulatory
Prostaglandin E_2	*via* EP3 receptor ↓	None alone	Stimulatory
Prostaglandin E_2	*via* EP4 receptor ↑	Inhibitory	Inhibitory
Prostaglandin E_1	*via* EP3 receptor ↓	None alone	Stimulatory
Prostaglandin E_1	*via* IP receptor ↑	Inhibitory	Inhibitory
Chemokines	↓	None alone	Likely stimulatory
β-adrenoceptors	↑	Inhibitory	Inhibitory
Cilostazol	↑	Inhibitory	Inhibitory
SSRI	↑	Inhibitory	Inhibitory
Magnolol, homokiol, curcumin	↑	Inhibitory	Inhibitory

molecule expression on activated platelets, as well as that of agents which interfere with the multiple receptor-ligand interactions involved in homotypic or heterotypic aggregation, represent exciting and promising strategies for future antithrombotic therapies. GPIIb/IIIa blockers are an emblematic example of this pharmacological approach. They have been demonstrated to prevent thrombotic events when given acutely and intravenously in patients with acute coronary syndromes and in those undergoing percutaneous coronary intervention, albeit at a price of increased risk of bleeding complications [134]. However, their counterpart oral GPIIb/IIIa inhibitors have shown no benefit or even increased mortality in clinical trials [135–137].

Interference in GPVI interaction with collagen has been also proposed as a viable antithrombotic strategy, and GPVI blocking agents are under development. Indeed, soluble GPVI dimers and anti-GPVI antibodies have been produced and tested in a variety of animal models [138–140]. However those tested to date appear to lack potency and efficacy and consequently *in vivo* data have not demonstrated a convincing antithrombotic effect. Fab fragment of anti-GPVI monoclonal antibody [141] and a novel GPVI-binding peptide [142] are currently undergoing evaluation in terms of their GPVI affinity and antithrombotic effectiveness in different species.

A novel and potentially attractive area of therapeutic development in this field is represented by so-called aptamers. Aptamers are protein-binding oligonucleotides that offer potential advantages over standard methods, in particular the possibility to combine a selective action against a specific protein–protein interaction with lack of antigenic activity (extensively reviewed by Keefe & Shaub [143]). Amongst the different antithrombotic aptamers that have been developed, inhibitors of vWF have shown good efficacy as antiplatelet agents in animal models, including non-human primates [144, 145], and have moved into the clinical setting with a first phase I trial [146] which has shown promising results.

Despite these considerations, the complexity of homotypic aggregation, whose main effector molecules vary depending on the haemodynamic conditions and specific features of the vascular lesions, may render it difficult to select a single ligand/receptor axis on which pharmacological modulation might result in a clear therapeutic antithrombotic action. On the other hand, interference with the adhesive interaction sustaining heterotypic complex formation may be more straightforward, given the critical role of P-selectin/PSGL-1 binding in mediating platelet-leucocyte aggregation. A number of different molecules have been identified or chemically synthesized with demonstrated ability to interfere with this binding. Heparin and heparin sulphate are ligands for P-selectin and block its binding to sialylated, fucosylated carbohydrate antigen related to sialyl Lewis^X (SLe^X), which is expressed on its ligands including PSGL-1. Several chemically-modified heparin derivatives (heparinoids) have been developed and have shown *in vivo* anti-inflammatory activity because of their blocking activity on P-selectin as well as L-selectin, the latter being expressed on leucocytes and endothelial cells [147]. More specific inhibitors of P-selectin such as monoclonal antibodies [148], SLe^X mimetics [149, 150] and recombinant PSGL-1 [151] have been produced and have demonstrated *in vivo* antithrombotic as well as anti-inflammatory effects in different animal models. However, the development of these compounds into effective drugs for clinical use has been greatly limited because of their relatively low selectivity amongst the different selectins, short circulating half-life, expense and potential antigenicity. In this context, apatmers offer a good therapeutic alternative. Indeed, P-selectin inhibitor aptamers are now available and have demonstrated higher binding affinity for P-selectin compared with SLe^X, and consequently a selective effect on PSGL-1 engagement. Moreover, little cross-interaction appears to occur with other selectins, including the L- and E-isoforms. It now needs to be established whether the *in vitro* data showing inhibition of heterotypic aggregation mediated by P-selectin aptamers

[152] will be supported by antithrombotic and anti-inflammatory effects *in vivo*.

Conclusions

We have here reviewed how the two main biological functions of platelet activation, namely thrombotic and inflammatory activity, can be differentially regulated in platelets. Examination of G_q- and G_i-dependent signalling pathways reveals that the combined activation of these two transduction cascades is necessary for effective thrombus formation, which requires the full platelet release reaction. However, regulation of G_i signalling alone, as occurs with pro-inflammatory cytokines, ADP and molecules involved in redox regulation, such as NO, appears to be crucially involved in alpha granule release, even in the absence of full platelet activation secondary to vascular damage. Pharmacological intervention on this molecular pathway, as provided by thienopyridine derivatives or cilastozol, as well as NO-potentiating agents, reduces heterotypic aggregate formation. Additionally, aptamers represent a novel and potentially effective means to inhibit platelet aggregation, especially of the heterotypic kind. However, in spite of rapid advances in our understanding of the molecular mechanisms regulating platelet function, further studies are required to define better the role of the different molecular effectors so far identified, as well as to identify novel potential molecular targets in order to develop more effective therapeutic strategies to correct platelet disorders.

Competing Interests

There are no competing interests to declare.

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