MINIREVIEW

Receptors that Activate Platelets (43251)

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The platelet plasma membrane is the focus of the interactions between the extracellular environment containing the agonists and the intracellular milieu in which the biochemical reactions responsible for cellular activation are located. These activation events are common to all agonists and include calcium mobilization, phosphorylation, formation of second messengers, and discharge of storage granules. Platelet receptors are externally oriented membrane proteins, accessible in the intact cell, that interact reversibly with specific agonists to form a complex, and then, in turn, initiate measurable intracellular responses. This review focuses on receptors for physiologically important agonists that stimulate intact platelets, including ADP, collagen, epinephrine, prostaglandin endoperoxides (or thromboxane A_2), and thrombin. This definition of a receptor implies that it binds the agonist reversibly and that the rate of binding must be consistent with the interaction of the cell with the agonist. The cellular response should be proportional to the concentration of receptor-agonist complex. Because the number of receptors is finite, binding to receptors should follow saturation kinetics. Finally, the receptor should display limited specificity.

Aggregin: An ADP Receptor Mediating Platelet Activation

The first agonist recognized for platelets was ADP. In 1972, Born (1) found that at low concentrations (0.1–0.5 μM) ADP induces human platelets to change shape from disks to spiculated spheres. At higher concentrations (2–5 μM), ADP induces platelet aggregation and secretion. An additional action of ADP was discovered 1 year later when Haslam (2) observed that ADP also antagonizes the stimulation of adenylate cyclase by prostaglandin E_1. This enzyme converts ATP to cAMP, an intracellular compound which inhibits platelet shape change, adhesion, aggregation, and release of granule contents (3). By inhibiting stimulated adenylate cyclase, ADP reduces cAMP, facilitating activation. The most traditional method of studying a receptor is to directly measure the binding of radiolabeled ADP to intact platelets. However, because ADP is hydrolyzed by platelet membrane phosphatases, the assumption that this represents equilibrium binding is not valid. More recently, studies by Jefferson *et al.* (4) have measured radiolabeled ADP binding to platelets fixed with formaldehyde. The results of these studies are not easily correlated with functional charges, however. Measuring the binding of ADP to isolated membranes is also not valid since it fails to distinguish proteins that were originally externally oriented and proteins that are usually found on the cytoplasmic face.

In 1978, we reported that the affinity labeling reagent 5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA) labels a single ADP receptor protein, aggregin, and inhibits its shape change in intact platelets (5). The structure of FSBA is similar to the structure of ADP and ATP in that it contains an adenosine group linked to an extended side chain through the 5′ position of the ribose. Sulfonyl fluoride, the reactive group, forms covalent bonds with nucleophilic amino acids with elimination of fluoride ion. Furthermore, [^{3}H]FSBA labels more than 40 proteins in adenosine nucleotide binding sites. When intact platelets are incubated with FSBA, only aggregin is labeled. FSBA inhibits ADP-mediated platelet shape change measured both spectrophotometrically and morphometrically (5, 6). In contrast, the corresponding guanosine analog had no effect, demonstrating the specificity of FSBA. The presence of ADP or ATP, but not of adenosine or GDP, completely prevented the radiolabeling.

On exposure to ADP, ^{125}I-fibrinogen at low concentrations bind to platelets proportionally to the concentration of fibrinogen (7, 8). When platelets are preincubated with FSBA and then exposed to ADP and

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fibrinogen, no fibrinogen is bound, and shape change and aggregation are totally inhibited (9).

The identification of the ADP receptor that mediates platelet activation as a 100-kDa protein, aggregin, raised two important questions. Was aggregin related to glycoprotein (GP) IIIa, also a 100-kDa protein, or to the receptor mediating the effect of ADP on adenylate cyclase?

The evidence indicates that GPIIIa and aggregin are different proteins. First, thrombasthenic platelets change shape normally in response to ADP (10). Second, FSBA incorporation was similar both qualitatively and quantitatively in thrombasthenic platelets lacking GPIIb/IIIa, as was the dose-dependent inhibition of ADP-induced shape change. Third, aggregin was not precipitated by murine monoclonal antibodies directed against the GPIIIb/IIIa complex or against GPIIIa, by a monospecific rabbit polyclonal antibody to immunopurified GPIIIa, or by a human antibody directed against an epitope present on GPIIIa (11, 12). Finally, it is known that aggregin and GPIIIa differ in molecular architecture because on reduction the electrophoretic migration of GPIIa decreases, whereas that of aggregin remains unchanged. These results indicate that these two receptors are distinct entities.

Aggregin is also not the receptor responsible for inhibition of adenylate cyclase (5). Preincubation with various concentrations of FSBA had no effect on the inhibition of prostaglandin (PG) I_2-stimulated adenylate cyclase by ADP. Neither the basal nor the stimulated levels of cAMP are affected by FSBA. Furthermore, 2-methylthio ADP is, on a molar basis, 75-fold more potent as an inhibitor of stimulated adenylate cyclase than as a stimulator of platelet aggregation. When platelets were incubated with 25 times the concentration of FSBA needed to block aggregation, no change in the number of binding sites for 2-methylthio ADP was detected. Moreover, the nonpenetrating thiol reagent, *p*-chloromercuribenzenesulfonate (pCMBS), failed to inhibit shape change induced by ADP, but reversed the effect of ADP in inhibiting cAMP accumulation (13).

Many antagonists of cell function may also serve as partial agonists, but require much higher concentrations, have incomplete effects, or both. We found that ADP induced shape change with a median effective concentration (EC_{50}) of 0.4 μM, whereas FSBA induced platelet shape change with an EC_{50} of 220 μM (14). A competitive antagonist of ADP, ATP inhibited FSBA-induced shape change. Thus, prior noncovalent binding of FSBA to platelets resulted in platelet activation, whereas covalent binding inhibited platelet response. Phosphorylation of the myosin light chain has been shown to correlate with ADP-induced shape change (15). High concentrations of FSBA that induce shape change also induce myosin phosphorylation (14), with a time course paralleling shape change. FSBA (50–200 μM) has also been demonstrated to increase platelet intracellular Ca^{2+}, confirming that it serves as a weak agonist (13). The inhibition of this response by pCMBS and the inhibition of ADP-induced Ca^{2+} mobilization by pCMBS but not FSBA (13) indicates that the action of FSBA to raise calcium is mediated by the adenylate cyclase receptor, strengthening the two-receptor hypothesis.

Collagen Receptors

No fewer than nine receptors on the platelet surface have been promulgated as the platelet collagen receptor. The first reason for these diverse conclusions is the polymorphic nature of collagen. There are at least 13 genetically distinct types of collagen, but not all of them react with platelets. The second difficulty is defining the functional event in platelet activation that is related to the putative collagen receptor. Early studies of the properties of collagen required to initiate platelet aggregation or secretion concluded that the collagen had to be in its native triple helical state (16, 17). Further studies showed that monomeric collagen molecules had to polymerize into fibrils to aggregate platelets (18, 19, 20, 21). Brown *et al.* (22) found that this requirement held for the earliest event of shape change induced by collagen. However, collagen-induced shape change, aggregation, and secretion are all secondary to induction of the release reaction, because collagen, like most agents that are capable of inducing platelet aggregation, will also cause the release of ADP from dense granules. We have utilized FSBA to inhibit ADP effects on platelets at the receptor level in an attempt to define the involvement of ADP in platelet activation by other effectors, including collagen.

The activation of platelets of collagen is thought to require both ADP and prostaglandin synthesis (23). When platelets were incubated with FSBA, the rate of aggregation by collagen was inhibited progressively over time. The result was not due to an effect of FSBA on prostaglandin synthesis. FSBA also inhibited fibrinogen binding stimulated by collagen. These results suggest that ADP is essential for platelet aggregation and fibrinogen binding is stimulated by collagen (24). Moreover, the amount of prostaglandin formed as a result of stimulation by collagen is not sufficient to cause shape change in the absence of ADP.

The search for the platelet receptor must, therefore, involve study of an earlier event than platelet shape change, namely, platelet adhesion to collagen. When vascular injury occurs, the platelets are observed to adhere to the collagen-containing basement membrane. One candidate is GPIV (IIIb), an 88-kDa major membrane glycoprotein. Tandon *et al.* (25) recently showed that this protein, previously identified as a receptor for thrombospondin (26), inhibits aggregation, secretion,

and shape change stimulated by collagen, without inhibiting platelet aggregation stimulated by thrombin, ADP, epinephrine, or arachidonate. Moreover, GPIV binds to type I collagen fibrils with a K_d value of 0.34 μM. The antigen binding fragments of monospecific polyclonal antibody to GPIV inhibited platelet adhesion to collagen in a microtiter plate assay. However, there is a second GPIV-independent mechanism that mediates subsequent anchorage of these adherent platelets to collagen.

Another candidate for a collagen receptor is a 65-kDa protein purified from detergent extracts of platelets by affinity chromatography on collagen by Chiang and Kanz (27). The isolated receptor, or an antibody to it, inhibits the binding of radiolabeled $\alpha 1$ (I) chain of collagen to platelets and inhibits the aggregation of platelets by type I collagen (28). Recently, these same investigators have shown that the antibody cross-reacts with two other proteins in solubilized platelet membranes with molecular masses of 90 kDa and 58 kDa (29). A monoclonal antibody to the 90-kDa protein also inhibited cleavage and ADP-induced aggregation, but not α-thrombin or epinephrine. The relationship of the 90-kDa protein to the 88-kDa GPIV is unknown.

Nieuwenhuis *et al.* (30, 31) recently described a patient with a mild bleeding disorder, an impaired collagen-induced platelet aggregation and adhesion, and a normal response to other agonists whose platelets had a deficiency of GPIa. The platelets failed to adhere and spread on exposed endothelium or on type III collagen at both low and high shear forces. A similar case has been reported by Kehrel *et al.* (32). In contrast, Rao *et al.* (33) have reported the case of a patient with immune thrombocytopenia whose platelets did not aggregate or secrete in response to collagen stimulation, but had normal responses to other agonists. The patient's serum immunoprecipitated an 88-kDa protein from ^{125}I-surface-labeled normal platelets identical to GPIV.

Santoro (34) observed that Mg^{2+} increased the adhesion of platelets to collagen 4- to 5-fold over that observed in the absence of divalent cations. Affinity chromatography yielded GPIa/IIa, a member of the superfamily of heterodimer adhesion receptors (35), VLA family. GPIa, a single component under reducing conditions, is actually two proteins separated under nonreducing conditions, GPIa and GPIa*. GPIa* is only surface labeled in activated platelets and appears to be a granule protein expressed on the activated platelet surface and in the supernatant of activated platelets (36). It is this glycoprotein that is missing in the two patients whose platelets failed to interact with collagen. This finding raises serious questions about whether GPIa or GPIa* is really a colalgen receptor. Thus, the nature of the collagen receptor remains unsettled at the present time.

Thromboxane A_2 Receptors

Thromboxane (TX) A_2 and its precursor, PGH2, are predominant metabolites of arachidonic acid in platelets (37) and potent inducers of activation, interacting with the same receptor (38). The identification of putative receptors has been difficult because antagonists of TXA_2, which induced activation of platelets, were also thromboxane synthetase inhibitors, and the participation of other agonists in the activation of TXA_2 confused the issue. For example, the role of released ADP in the action of TXA_2 has been controversial. In TXA_2 transfer experiments, ADP appeared to be important in the observed responses (39). Further evidence is provided by the inhibition of platelet aggregation by the endoperoxide analogues U46619 and azo-PGH_2 by FSBA (40); however, neither FSBA nor ADP-metabolizing enzymes could block shape change induced by prostanoid derivatives, a response most proximate to binding to the receptor.

Three separate developments will allow a closer approach to the eventual identification and isolation of the TXA_2 and prostaglandin endoperoxide receptor. First, a competitive antagonist, 9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13, 14-dihydro-13aza-15$\alpha\beta$-ω-tetranor-TXA_2 (PTA-OH) (41), has been developed and labeled with ^{125}I. Scatchard analysis of equilibrium binding yielded a B_{max} of 1.8 pmol/mg protein and a K_d value of 30 μM. Second, a series of TXA_2/PGH_2 antagonists inhibit the binding of PTA-OH with a potency correlated with their action as inhibitor of platelet aggregation. A related photoaffinity analogue, a diazonium salt of PTA-OH, on photolysis decreased the total binding by PTA-OH without affecting the K_d (42). FInally, an extremely specific TXA_2 receptor antagonist, S-145, (±)-5(*Z*-7-[3-*endo*-phenylsulfonylamino[2.2.1]bicyclohept-2-*exo*-yl] -heptanoic acid, suppressed U46619 shape change as well as collagen-induced shape change (43). The latter has been shown to absolutely require TXA_2 because, although low azo-PGH_2 requires ADP (as shown by FSBA inhibition), at high concentrations azo-PGH_2 can overcome FSBA inhibition of shape change (40). Formation of TXB_2 is not affected by S-145, but has a K_i of 2.5 nM for inhibition of the binding of [^{3}H]-U46619 to human platelet membranes. These compounds should ultimately allow characterization of this important receptor.

α_2-Adrenergic Receptor

Interaction of platelets with epinephrine results in several responses, including platelet aggregation, exposure of fibrinogen receptors (7), and inhibition of adenylate cyclase activity (44). These responses are coupled with the increase in Ca^{2+} uptake (45) with a concomitant rise in intracellular ionized calcium. Responses to epinephrine by the platelets are mediated by α_2-adre-

nergic receptors (46), one of four subtypes of adrenergic receptors. Like β_1- and β_2-adrenergic receptors, α_2 is linked to adenylate cyclase, but unlike them, it inhibits the enzyme and is coupled to the N_i type of G protein that is regulated by the balance between guanosine 5′-triphosphates and guanosine 5′-diphosphates. The α_2-receptors are distinguished from the β-receptors by a much greater reactivity to epinephrine and norepinephrine than to isoproternol. The α_2-receptors have yohimbine as a specific antagonist (47). Thus, binding of ^{3}H-methyl-yohimbine (48) has been used to measure the number of α_2-receptors (B_{max} = 270/platelet) and the dissociation constant (K_d = 2.5 nM). The platelet α_2-adrenergic receptor has been purified 80,000-fold to homogeneity (49) and is a single polypeptide, M_r = 64,000. Verification of the identity of the receptor was achieved by covalently labeling it with the alkylating affinity analog [^{3}H]phenoxybenzamine and demonstrating that the proper order of the potency of both agonists and antagonists was obtained. The receptor has an essential thiol group because it can be inhibited by phenylmercuric chloride and reactivated by dithiothreitol. Using a partial amino acid sequence from the purified receptor as a guide, the receptor was cloned from a human genomic library (50). Like the other adrenergic receptors, no introns existed in the α_2-adrenergic gene. The gene coded for a 450-amino acid protein with seven clusters of hydrophilic residues that probably represent the membrane spanning domains. The amino terminus and three loops extend extracellularly, whereas the carboxy terminus and three additional loops are cytoplasmic. The third cytoplasmic loop contains a consensus sequence for phosphorylation of a serine or threonine by protein kinase A. Mehta *et al.* (51) correlated the EC_{50} of epinephrine to induce a primary wave of aggregation and the EC_{50} of epinephrine to inhibit yohimbine binding. There was no relationship of the B_{max} or K_d of yohimbine binding itself.

Whether epinephrine by itself can aggregate platelets or simply potentiates the effects of other agonists has been controversial. The increase of Ca^{2+} and antagonism of adenylate cyclase activity are not sufficient to induce aggregation. When platelets were washed in the presence of prostacyclin and albumin and resuspended in a buffer containing apyrase, no response was elicited by 1–10 μM epinephrine (52), indicating the requirement for ADP. Shattil *et al.* (53) found that diluted blood in the presence of hirudin and apyrase still showed a response to epinephrine as assessed by flow cytometry, although it was decreased by 50%. Because the K_m of apyrase is about 0.1 mM for ADP, it is hard to remove small concentrations (<0.1 μM) of ADP that are known to synergize the response to epinephrine. Accordingly, our laboratory (54) employed FSBA to render the platelet insensitive, even to submicromolar quantities of ADP. When platelets were incubated with FSBA, epinephrine-induced aggregation and exposure of fibrinogen binding sites were progressively inhibited until epinephrine produced no response even at 10 μM. These results indicated the absolute dependence of epinephrine-induced platelet aggregation on the ADP receptors. In contrast, FSBA did not change the number or affinity of α_2-adrenergic receptors as measured by [^{3}H]yohimbine binding. Although epinephrine did not alter the number of ADP binding sites as measured by ^{3}H-FSBA, it did increase the affinity of binding of ADP by a factor of 10.

Alteration in the number or affinity of α_2-adrenergic receptors of platelets has been documented in essential thrombocythemia (55). Decreased α_2-adrenergic receptors have been documented in congestive heart failure and coronary artery disease (56), and α_2-adrenergic receptors are increased in anorexia nervosa (57). It is not clear, however, whether a decrease in the number or affinity of receptors for epinephrine is due to actual decreased regulation or merely represents retained agonist (58). Recently, we described a family (59) whose members had selective impaired aggregation and secretion responses to epinephrine. The number of α_2-adrenergic receptors measured by [^{3}H]yohimbine binding was 13–36% of normal. Despite the impaired aggregation response to epinephrine, the platelets for this family showed normal inhibition by epinephrine of PGI_2-stimualted adenylate cyclase, indicating that fewer receptors are needed for this response than that of platelet aggregation.

Thrombin Receptors

Thrombin can activate platelets by multiple pathways. At low concentrations of thrombin ($\leq$ 1 nM), secretion of ADP, serotonin, and fibrinogen from platelet dense and α-granules may contribute to its action. At moderate concentration (2 nM), thrombin, unlike collagen, epinephrine, and prostaglandin endoperoxides, stimulates platelets by an ADP-independent mechanism (60). There are two corresponding types of receptors (61), each coupled to different platelet responses. The first type (R1) is mediated by a small number (50 sites/platelet) of high affinity (K_d = 0.3 nM) (62), and mediates the inhibition of stimulated adenylate cyclase, the secretion of acid hydrolases, and the activation of phospholipase A_2. Responses coupled to R1 require continuing occupation of the receptor (63, 64), but only low concentrations of thrombin (0.5–1 nM), which are probably dependent on Na^+/H^+ exchange. This dependency leads to cytoplasmic alkalinization because the pathways are blocked by amilorides (65, 66). This pathway is not mediated by γ-thrombin, seems to have the characteristics of an agonist-receptor equilibrium, and may involve binding to GPIb, since in Bernard-Soulier platelets, thrombin binding is diminished (67) and monoclonal antibodies to GPIb also block throm-

bin binding (68). The stoichiometry of the reaction remains in doubt because only 50 molecules of thrombin bind to the high affinity site, yet 34,000 molecules of GPIb exist on the intact platelet (69).

The second type (R2) is mediated by an intermediate number of binding sites (1,700/platelet) with moderate affinity (K_d = 11 nM). This receptor is linked to phospholipase C, which cleaves phosphatidyl bisphosphate to diacetylglycerol and inositol triphosphate. Diacetylglycerol stimulates protein kinase C and is closely linked to secretion, whereas inositol triphosphate plays a major role in increasing intracellular calcium. These responses require only transient exposure to thrombin (10–20 sec) in moderate concentrations (greater than 2 nM) and result in platelet aggregation and exposure of fibrinogen binding sites. These responses are more typical of an enzyme-catalyzed reaction and can be mediated by γ-thrombin.

We have detected a platelet membrane protein (100 kDa) that is cleaved after exposure of washed intact platelet to thrombin: aggregin, an ADP receptor (60). If thrombin is incubated with platelet membranes containing aggregin that has been labeled by ^{3}H-FSBA, or if intact platelets are depleted of ATP, no cleavage occurs (70), unlike GPV, which is directly cleaved by thrombin on platelet membranes. These findings suggest that thrombin may act indirectly by activating some other intracellular proteolytic enzyme to hydrolyze aggregin. This response was not blocked by a variety of serine protease inhibitors, but was inhibited by thiol protease inhibitors, leupeptin, and antipain, at a concentration (20 μmol) that does not inhibit thrombin enzymatic activity. These results suggested that a thiol protease cleaved aggregin. We showed that purified platelet calpain (a calcium-activated thiol protease), unlike thrombin, is able to cleave aggregin in the platelet membrane or in intact platelets, because thrombin can raise intracellular calcium and thus activate calpain. Calpain will aggregate washed platelets in the presence or absence of FSBA if fibrinogen is added, suggesting that the thrombin-induced platelet aggregation and the exposure of fibrinogen binding sites are mediated through the activation of calpain (70), independent of ADP. Consistent with the hypothesis that calpain mediates thrombin-induced platelet activation, high molecular weight kininogen (HK), the most potent plasma inhibitor of platelet calpain (71), was found to inhibit thrombin-induced aggregation and cleavage of aggregin (72). The inhibition was a function of the concentration of HK, and occurred in platelets modified by FSBA. Calpain is exposed on the surface of platelets stimulated by thrombin, as shown recently by immunofluorescent studies (73).

About 10 times as much thrombin is needed to stimulate platelets in plasma as washed platelets. This difference has now been attributed to the presence of HK, because plasma deficient in HK behaves similarly to washed platelets, and HK can itself increase the requirement for thrombin over that of normal plasma. Thus, HK appears to modulate thrombin action in normal plasma (72), both by inhibiting calpain and by preventing thrombin binding to platelets. This inhibition displays specificity since HK inhibits both α- and γ-thrombin but fails to inhibit aggregation by ADP, collagen, a calcium ionophore, A23187 (without added Ca^{2+}), the protein kinase C stimulator, phorbol myristate acetate, a combination of the latter two, or the stable prostaglandin endoperoxide, U46619. Moreover, HK does not affect platelet cAMP levels or inhibit thrombin enzymatic activity. The nature of the intermediate affinity receptor is not clear, but additional sites on GPIb is a reasonable, working hypothesis.

Summary

This review highlights the increasing knowledge of the biochemistry, pathology, and cell and molecular biology of platelet receptors. A receptor for ADP has been identified using the affinity label FSBA as aggregin, a 100-kDa membrane protein responsible for shape change, aggregation, and exposure of fibrinogen binding sites. A variety of putative receptors for collagen have been described, with GPIa/IIa and GPIV receiving the most attention recently. A thromboxane A_2 receptor has been identified using receptor antagonists and photoaffinity labels. The α_2-adrenergic receptor has been cloned and expressed. The platelet thrombin receptor has been tentatively identified as GPIb. Following binding of thrombin to this receptor, activation of calpain occurs, with cleavage of aggregin leading to exposure of GPIIb/IIIα and platelet aggregation. Isolation, expression, or both of the ADP, collagen, and thrombin receptors as single gene products of the human platelet responsible for activation, and more complete understanding of stimulus-response coupling, should allow for greater specificity of drugs with selective therapeutic actions.

Note added in proof. Since this review was submitted, the human platelet thrombin receptor has been identified by expression cloning (74) and belongs to the family of peptide receptors.

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