MINIREVIEW

High and Moderate Affinity Pathways for α-Thrombin-Induced Platelet Activation

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Abstract. **The interpretive review represents evidence for two pathways of α-thrombin-induced activation in human platelets and evaluates the possible roles of a functional thrombin receptor recently identified by expression cloning.** [P.S.E.B.M. 1991, Vol 198]

Alpha-thrombin is a potent platelet-activating agent and can cause full aggregation and secretion at concentrations as low as 0.04 units/ml (0.4 n*M*). Proteolytically active thrombin is required for platelet activation, but the kinetics are inconsistent with simple proteolysis. In a proteolytic process, the *rate* is proportional to enzyme concentration, while the *extent* is independent of it. Studies both by microcalorimetry (1) and by examining the kinetics of Ca^{2+} and ATP secretion (2–6) have shown that platelet activation by α-thrombin has activation kinetics characteristic of both an enzyme-catalyzed reaction and an agonist-receptor equilibrium. Steady state binding studies have shown that platelets contain two different types of binding sites within the physiologically relevant range of α-thrombin concentrations: high affinity sites (K_d 0.3 n*M*; 50 sites/platelet) and moderate affinity sites (K_d 11 n*M*; 1,700 sites/platelet) (7–9). Platelets also have a low affinity site (K_d 2 μ*M*; 600,000 sites/platelet), as identified by LIGAND analysis, which may correspond to the nonspecific binding identified by other investigators using graphical techniques. When platelets are treated with *Serratia marcescens* metalloprotease, high affinity binding is lost and only the moderate and low affinity binding sites are detectable (9).

In the absence of the high affinity sites, platelets still retain their ability to mobilize Ca^{2+}, change shape, secrete, and aggregate, although they show an attenuated response and require about 20-fold higher α-thrombin concentrations. The α-thrombin concentrations required for half-maximal activation of intact and Serratia-treated platelets are 0.35 n*M* and 10 n*M* (9, 10), values in good agreement with the dissociation constants of the high and moderate affinity sites. However, it has been suggested that platelet activation could be mediated through the low affinity or nonspecific binding sites (11). This appears unlikely, since only about 1% of the low affinity sites would be occupied at 0.5 n*M*, the α-thrombin concentration causing full activation, but this would correspond to about 6000 sites and could be sufficient if the determining factor is not the percentage of receptor occupancy but the total number of sites occupied.

These data suggesting that there are two physiologically relevant platelet receptors are supported by functional studies: platelet responses, such as aggregation, α- and dense granule secretion, and the activation of phospholipases A_2 and C, differ in their sensitivity to chymotrypsin (12), in their requirements for Na^+ ions (13–15), in their requirements for receptor occupancy (16–18), and in the role of nucleotide regulatory proteins (19, 20). Moreover, at low, but not high, concentrations of α-thrombin, secretion from α-granules is required for aggregation (21), while aggregation and secretion are both sensitive to cyclooxygenase inhibitors

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(22–24). Furthermore, high, but not low, concentrations of α-thrombin induce aggregation in platelets from patients with storage pool disease (25), and this aggregation is insensitive to added thrombospondin (26).

On the basis of these and other studies, it is possible to propose a two-receptor model for the mechanisms involved in the activation of platelets by α-thrombin as shown in Figure 1.

High Affinity Receptor Pathway

The most direct evidence that glycoprotein (GP) Ib is the high affinity receptor for α-thrombin, and not merely a thrombin binding site, comes from studies showing that several monoclonal antibodies directed against GPIb are able to inhibit thrombin-induced platelet activation (27–30). Recent studies from our laboratory have shown that there are GPIb-dependent and GPIb-independent pathways of thrombin-induced platelet activation and that this inhibition by anti-GPIb monoclonal antibodies arises specifically through the high affinity pathway (31). TM60, a monoclonal antibody directed against the thrombin-binding domain in GPIb, completely blocked the ristocetin-dependent binding of von Willebrand factor to GPIb in intact platelets, but inhibited only to 50% the binding of α-thrombin and the binding of von Willebrand factor to GPIIb/IIIa resulting from thrombin-induced activation. In platelets treated with *S. marcescens* protease to remove GPIb, a concentration of TM60 in large excess of the maximum possible remaining copies of GPIb failed to inhibit platelet activation, indicating a second pathway independent of GPIb (Fig. 2).

These studies show that platelet activation by α-thrombin is mediated through both GPIb-dependent (high affinity) and GPIb-independent (moderate affinity) pathways. Further evidence that GPIb is the high affinity receptor comes from findings that neither high affinity binding nor platelet response at a low α-thrombin concentrations is detectable following specific cleavage of the glycocalicin portion of GPIb with *S. marcescens* protease (9, 10), and similar effects are seen in platelets from which GPIb has been removed by treatment with chymotrypsin (32, 33) or elastase (34, 35). There are concomitant decreases in GPIb, thrombin-responsiveness, and high affinity thrombin binding in Bernard-Soulier platelets (32) and in platelets from patients with myeloproliferative disorders (36, 37).

Although GPIb meets the requirements for a high affinity thrombin receptor, it is not itself susceptible to thrombin digestion. It may, therefore, act through a classical receptor-mediated mechanism or, alternatively, the binding of α-thrombin to GPIb may orient it correctly toward a separate proteolytic substrate (32, 38, 39; Fig. 3). Furthermore, since platelets devoid of GPIb continue to be able to be activated, although they require much higher concentrations of α-thrombin, it is not clear whether GPIb functions as a receptor in its own right or whether it serves to sensitize platelets to activation induced through the moderate affinity pathway; this important question cannot, however, be answered until methods are developed for the specific inhibition of the moderate affinity receptor.

GPIb, GPIX, and GPV are all absent in Bernard-Soulier platelets (40, 41). In the resting normal platelet, GPIb is tightly complexed with GPIX in a 1:1 stoichiometric ratio (42) and this complex interacts with an actin-binding protein dimer (43); it may be noted that

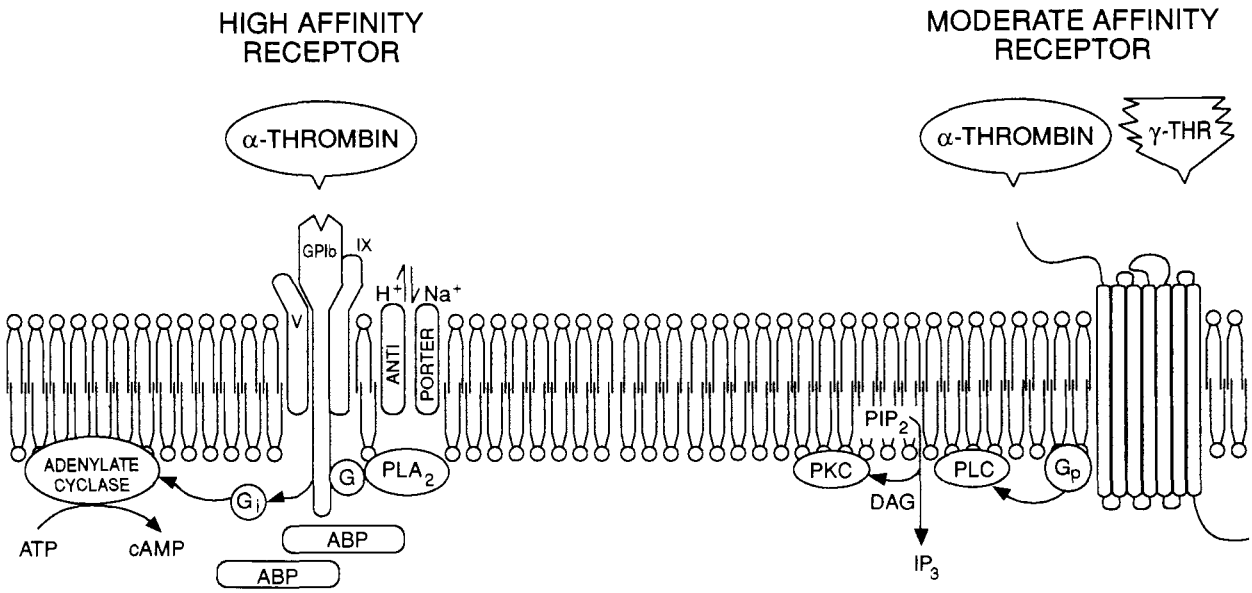

Figure 1. A model for the high and moderate affinity pathways of platelet activation by α-thrombin.

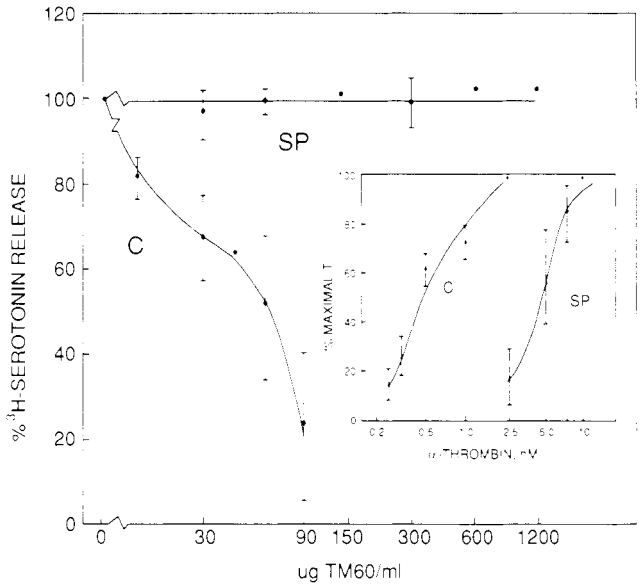

Figure 2. Effect of TM60 on [^{3}H]-serotonin release from intact and Serratia-treated (SP) platelets. To determine its effect on [^{3}H]serotonin release, TM60 was preincubated with platelets (5 min) before the addition of 0.5 n*M* α-thrombin to control (C) and 2.5 n*M* α-thrombin to Serratia-protease-treated platelets. Release was measured and is shown normalized to 100% (i.e., without the addition of TM60) to compensate for variations in total secretion between the six individual donors examined. Release at these concentrations of thrombin was 67 ± 7 (C) and 64 ± 13 (SP). Concentrations of TM60 as high as 1200 μg/mL were without effect on the release of [^{3}H] serotonin from Serratia-treated platelets. Inset: Dose-response curves for the maximal release of [^{3}H]serotonin from intact platelets (C) and Serratia-treated platelets (SP) versus thrombin concentration. Reprinted with permission from *Blood* (31).

the molecular weight of this extended complex of GPIbα, GPIbβ, GPV, GPIX, and actin-binding protein dimer (726,000) is identical to the functional molecular weight of the high affinity thrombin receptor determined by radiation inactivation using intact platelets (720,000 ± 160,000; Ref. 7). The difference between the number of copies of GPIb on platelets (n = 28,000) and the number of copies of the high affinity receptor (n = 50) may reflect a requirement for the formation of this extended complex for high affinity binding.

GPV is apparently not tightly complexed to GPIb/GPIX. Although it is the only glycoprotein known to be susceptible to thrombin digestion in intact platelets (44, 45), it is not involved in thrombin-induced activation based on the lack of effect of its proteolytic removal (46, 47), the lack of correlation of GPV hydrolysis and activation by α- and γ-thrombins (48), and the absence of inhibition of activation when proteolysis is blocked by anti-GPV antibody (49).

Adenylyl cyclase, coupled directly or indirectly through G_i, may also be considered to be in the high affinity pathway, based both on the inability of α-thrombin to depress stimulated cAMP levels in platelets treated with chymotrypsin (12) or Serratia protease (unpublished) and the fact that both basal and stimulated levels of cAMP are maximally inhibited by <1 n*M* α-thrombin (50).

Arachidonate release occurs at low thrombin concentrations (51) and is not seen in chymotrypsin-digested platelets that lack GPIb (12). The enzymes of arachidonate generation are, therefore, probably associated with the high affinity pathway. In platelets, a phospholipase A_2 (PLA_2) active against phospholipids appears to be the main source of arachidonate (52), but another source is phospholipase C/1,2-diacylglycerol lipase (53, 54). Moreover, platelets may contain two types of PLA_2: one degrades phosphatidylethanolamine and phosphatidylcholine and requires alkaline pH and millimolar concentrations of Ca^{2+} (52), whereas another PLA_2, identified in a particulate fraction of horse platelets, is specific for phosphatidic acid, has a pH optimum of about 7.0, and requires low concentrations of Ca^{2+} (10 μM; 55). This second, phosphatidate-spe-

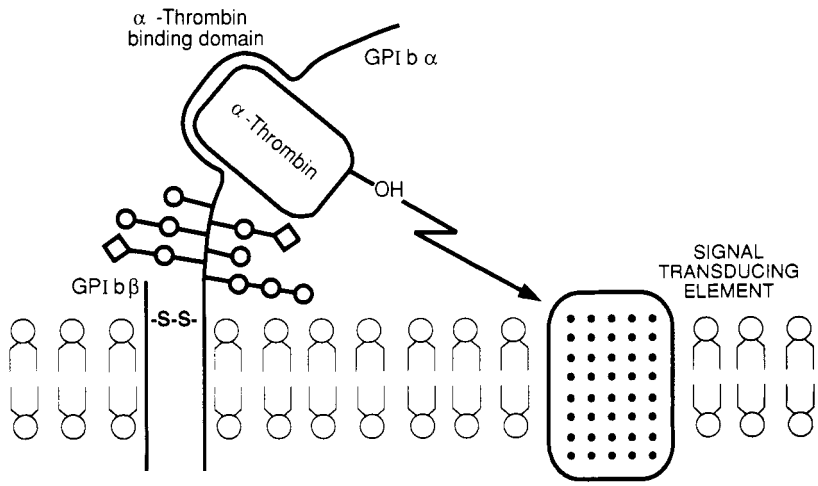

Figure 3. A possible mechanism for the role of GPIb in the high affinity α-thrombin pathway. In this model, α-thrombin binds to a specific site on GPIb in the high molecular weight receptor complex and is oriented to interact proteolytically with another component.

cific PLA_2 is particularly interesting in view of our findings that platelet stimulation by low concentrations of α-thrombin results in a slight reduction of the pH of the platelet cytoplasm from resting levels of 7.2 (10). PLA_2 appears to be coupled to the receptor through an as-yet-unidentified G protein (56–58).

α-Thrombin inhibits [^{32}P]ADP ribosylation of pertussis toxin substrates in saponin-treated platelets by >90%, probably due to changes in Gα conformation (59–61). The thrombin concentrations used in these studies have generally ranged from 1 unit to 10 units/ml; even at the lower of these concentrations, moderate affinity receptors would be ~50% occupied and high affinity receptors 97% occupied. Thus, it is not surprising that these studies have not clearly resolved the question of whether G_i and G_p are different G proteins or the same protein performing two functions, nor have they defined the possible role of the unidentified pertussis-sensitive G protein that is thought to mediate the activation of PLA_2 in platelets (56, 57), possibly through the $\beta\gamma$-subunit (62–64). A $G_{z\alpha}$ subunit has been identified in platelets that is not a substrate for pertussis toxin, but undergoes phosphorylation in intact or permeabilized platelets in response to α-thrombin (63). However, because of the α-thrombin concentrations used (1 unit/ml), it is not possible to determine whether it is part of the high or moderate affinity pathways.

PLA_2 may also be coupled to a Na^+/H^+ antiporter that is activated at low thrombin concentrations (<0.4 nM) (13–15) corresponding to the high affinity pathway.

Moderate Affinity Pathway

Until now, the moderate affinity receptor has been much more poorly defined, largely because of the absence of methods for its selective removal or inhibition. The functional molecular size of the moderate affinity binding site is only 30,000, as determined by radiation inactivation (7), although the structural molecular weight could be higher depending on subunit structures, disulfide bonding, and carbohydrate content.

In the original version of this model (65), it was tentatively suggested that the thrombin-binding protein in the moderate affinity receptor might also be GPIb, based on the presence of both high and moderate affinity binding sites on purified GPIb and glycocalicin (66). However, our recent studies (31) have shown that the moderate affinity receptor is immunologically distinct from GPIb.

Phospholipase C (PLC) and protein kinase C may be assigned to the moderate affinity receptor pathway, since chymotrypsin-treated platelets, which lack GPIb, continue to exhibit these activities in response to α-thrombin at higher concentrations and both enzymes are stimulated by γ-thrombin, which has proteolytic activity but does not bind saturably to platelets (12). The association of proteolytic events with PLC activation has also been suggested from studies with leupeptin (67). Since inositol triphosphate is thought to induce the release of Ca^{2+} from the dense tubular system, the coupling of PLC to the moderate affinity pathway could be the major source of thrombin-induced elevations in cytoplasmic Ca^{2+} concentrations.

Taking the model as a whole, it may be noted that those activities which have been shown to require continuous receptor occupancy, such as arachidonate mobilization and the formation of phosphatidic acid (16, 17), are mediated through the high affinity pathway and probably represent a receptor-mediated mechanism. On the other hand, the activities that require only transient receptor occupancy, such as the synthesis and breakdown of polyphosphoinositides, correspond to the moderate affinity pathway, which may be linked to substrate proteolysis. The above model indicates that, in intact platelets at physiological α-thrombin concentrations greater than 0.4 nM, platelet activation involving both the high and moderate affinity pathways would have the required kinetic properties of both receptor-mediated and enzyme-catalyzed reactions.

We have recently established that the rapid mobilization of Ca^{2+} that is observed in intact platelets results from the synergistic interaction of the two pathways and occurs only when both high and moderate affinity sites are occupied (68). These results are in agreement with recent continuous-flow kinetic studies of rapid $[Ca^{2+}]_i$ dynamics in platelets activated by the thrombin analogs, γ- and diisopropyl fluorophosphate-treated α-thrombin (69). These studies demonstrated that, although the high affinity-binding activity of thrombin is not required for a major increase in cytoplasmic Ca^{2+} concentration, fully active α-thrombin acting at both sites induces a more rapid (≤2 sec) mobilization of Ca^{2+}. It was further suggested that the earliest Ca^{2+} mobilization in response to α-thrombin involves a common mechanism, different from PLC and inositol triphosphate, which is at present unidentified (70). Taken together, these results suggest that whereas the moderate affinity pathway acts by means of PLC and inositol triphosphate to mobilize large stores of Ca^{2+}, a more rapid mobilization, possibly from this unidentified intracellular site, is evoked by the synergistic response of the high and moderate affinity pathways.

The results summarized here suggest the following sequence of events. Under conditions in which only very low concentrations of α-thrombin are generated (<0.4 nM), there would be inhibition of basal adenylyl cyclase activity and activation of PLA_2 accompanied by a relatively slow rate of increase in cytoplasmic Ca^{2+} concentration. As the amount of thrombin generation increased above 0.4 nM, PLC would become activated and there would be a synergistic increase in the rate of cytoplasmic Ca^{2+} accumulation. This synergism may

arise from the contribution of products of the high affinity pathway that act prior to or during the activation of PLC so as to increase its rate, or they may act subsequent to PLC activation to inhibit the degradation of phosphoinositides or other pathways involved in the release of Ca^{2+} from the dense tubular system.

Molecular Biology

In an important and elegant recent publication, Dr. Shaun Coughlin and colleagues (71) describe expression of a functional thrombin receptor in frog oocytes following microinjection of size-fractionated mRNA from the human erythroleukemia cell line and the DAMI megakaryocytic cell line. The mRNA encoding this receptor was detected in platelets and endothelial cells using the polymerase chain reaction (71). A hydropathy plot of the deduced amino acid sequence showed the receptor to be a member of the seven-transmembrane domain receptor family. The amino-terminal domain contains the sequence LDPRS, which resembles the thrombin-sensitive site in protein C. It has been proposed that thrombin's mechanism of action at this receptor is to cleave the R41-S42 peptide bond, revealing a new amino terminus which could function as a tethered ligand. This hypothesis has been supported by demonstrating that the peptide corresponding to the new amino terminus created by thrombin's action is able to induce the aggregation and secretion of washed platelets. These important studies should greatly facilitate elucidation of the mechanisms of thrombin-induced platelet activation.

So far, it has not been determined whether the cloned receptor corresponds to the high or moderate affinity pathways. Tentatively, it may be assigned as initiating the moderate affinity pathway, since its molecular weight deduced from its amino acid sequence (~42,000) is in the region of the functional molecular size of the moderate affinity receptor (30,000 ± 9,000), as deduced from radiation inactivation and target analysis (7), and it interacts synergistically with the high affinity receptor to induce platelet activation (unpublished).

The DNA for the high affinity receptor GPIbα has also been cloned (72), but the deduced structure does not shed light on the role of GPIb in transducing the signal of thrombin interaction. However, an acidic sequence found in GPIbα, D_{277}YYPEEDTEGD, may correspond to an acidic sequence, EPFWEDEEKNES, in the putative moderate affinity receptor that has homology to an acidic region in the carboxyl tail of hirudin (DFEEIPEE) that binds to the anion exosite of α-thrombin (73, 74). That this acidic domain corresponds to the thrombin-binding site of GPIbα is supported by the fact that monoclonal antibodies with epitopes at or near this sequence inhibit thrombin binding and platelet activation (31) and that synthetic peptides derived from the primary sequence of GPIbα near this acidic region inhibit thrombin-induced platelet activation and the binding of the monoclonal antibody TM60 (75), which is an antibody that inhibits platelet activation through the high affinity pathway (31).

Thrombin Purity

The homogeneity and specific activity of the thrombin preparations used in platelet activation studies are of fundamental importance. This may appear to be self-evident, but the specific activity is frequently not cited or, when cited, has corresponded to a purity as low as 5% (76). Homogenous human α-thrombin has a specific activity of 3000 NIH units/mg, based on fibrinogen clotting assays (77). Less pure preparations may be contaminated with β- and γ-thrombins, which do not clot fibrinogen but which do have the capacity to induce platelet activation; that is, these preparations could have a much greater platelet-activating capability than is implied from their fibrinogen clotting activity. Moreover, in many cases, thrombin concentrations have been used (e.g., 10 units/ml) that are far in excess of the concentrations required to induce full platelet activation. These supraoptimal concentrations of thrombin may induce metabolic changes that are not intrinsic to activation mechanisms, but that are merely epiphenomena resulting from the overstimulation of platelet metabolic pathways.

These observations may explain the uncertainty regarding the changes in cytoplasmic pH arising during platelet activation. Many investigators have reported cytoplasmic alkalinization or a transient acidification followed by alkalinization. We have found that cytoplasmic acidification of ~0.06 pH units occurs at the minimal α-thrombin concentrations that result in full platelet activation (0.4 n*M*), but that incrementally higher α-thrombin concentrations lead to a progressive cytoplasmic alkalinization (10). Similarly, we have recently found that the rate of increase in cytoplasmic Ca^{2+} concentrations is low when only the high or moderate affinity receptors are occupied, but that there is a synergistic increase in rate when both receptors are significantly occupied (68). Taken together, these two sets of observations are consistent with the conclusion that cytoplasmic alkalinization is not required for Ca^{2+} mobilization (78, 79).

As well as the purity of α-thrombin, it is necessary to ascertain that derivatized thrombins are free of residual proteolytic activity as measured with the chromogenic substrate S2238, since high concentrations are required in antagonist studies. α-Thrombin derivatized with diisopropyl fluorophosphate is known to contain residual proteolytic activity (80). We have recently shown (10) that thrombin derivatized with tosyl-lysine chloromethylketone exists as an equilibrium mixture

containing 4% α-thrombin activity, as measured with S2238, and is, therefore, unsuitable for use as an antagonist. However, α-thrombin derivatized with phenylalanine-proline-arginine chloromethylketone (PPACK-thrombin) did not contain α-thrombin activity above the detectable minimum of 0.1 nM at a 500 nM-PPACK-thrombin concentration, or 0.02%. PPACK-thrombin is a specific inhibitor of platelet aggregation and secretion by α-thrombin, but has no effect on activation induced by ADP, collagen, epinephrine, ristocetin, or arachidonate. While we had originally been unable to detect inhibition of shape change by PPACK-thrombin, we have subsequently found that this inhibition does occur at very low concentrations of α-thrombin (0.1 nM; 0.01 units/ml) and requires a PPACK-thrombin concentration of 500 nM. These results show that PPACK-thrombin binds tightly to the high affinity receptor but is a weak antagonist of platelet activation. This may imply that the platelet-binding domain in PPACK-thrombin is unaffected by derivatization, whereas the active-site domain may be distorted by the bulky hydrophobic substituent.

Several conclusions emerge from the studies reviewed here: (i) the available evidence has permitted a testable hypothesis to be proposed for a two-receptor model for platelet activation by α-thrombin; (ii) GPIb participates in activation as the high affinity receptor and the recently cloned thrombin receptor may initiate the moderate affinity pathway; (iii) the two pathways may interact synergistically so that stimulation of both pathways is required for optimal platelet activation; and (iv) future studies should utilize α-thrombin preparations of a high degree of purity and at concentrations that are compatible with the affinities of the two-receptor pathways.

This review has attempted to define, at least in part, high and moderate affinity pathways mediating platelet activation by α-thrombin. However, this model may also be applicable to eukaryotic cells that are stimulated by α-thrombin. The molecular weight (~150,000) of a putative thrombin receptor identified in fibroblasts by cross-linking studies (81) and immunoprecipitation (82) is comparable to that of GPIb, and a molecule immunologically related to GPIb has been reported in endothelial cells (83), which are themselves stimulated by thrombin. Moreover, optimal proliferative responses are thought to involve the synergistic interaction of proteolytic and nonproteolytic events (84), and a two-receptor model has been proposed for thrombin stimulation in fibroblasts (85) that has similarities to that proposed here for platelets.

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