“Thrombin” Receptor-directed Ligand Accounts for Activation by Thrombin of Platelet Phospholipase C and Accumulation of 3-Phosphorylated Phosphoinositides*

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Using three experimental approaches, we have addressed the questions of whether the presence of saturably bound thrombin plays a role in potentiating the activation of platelet phospholipase C (PLC) and/or accumulation of the 3-phosphorylated phosphoinositides (3-PPI), i.e. phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, and whether the generation of tethered ligand (Vu, T.-K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 1057–1068) by thrombin can account fully for thrombin’s proteolytic effects in activating platelets, as gauged by the above parameters. We have 1) measured PLC activation or 3-PPI after we have exposed platelets to thrombin for various periods and either blocked thrombin’s proteolytic activity without interrupting its binding or blocked both binding and proteolytic activity of thrombin; 2) attempted to potentiate 3-PPI accumulation, using combinations of protein kinase C stimulation, Ca2+ elevation, and saturating but proteolytically inactive thrombins; and 3) compared the activation of platelets by thrombin with activation by the “thrombin” receptor-directed peptide, SFLLRNPNDKYEPF (SFLL; a portion of the N-terminal tethered ligand at the cell surface is thought in this setting been examined. Such studies are also presented here.

We conclude that the initial and sustained effects of thrombin in stimulating PLC and the accumulation of 3-PPI are completely attributable to thrombin’s proteolytic activity. Further, thrombin’s effects in promoting these responses can be accounted for by the actions of SFLL peptide, and by implication, formation of tethered ligand.

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Thrombin is one of the most potent natural agonists known for platelets. It rapidly activates phospholipase C (PLC)1 (1, 2) and, in a manner dependent in part upon the activation of PKC (3), stimulates the accumulation of PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (4, 5), collectively referred to as “3-phosphorylated phosphoinositides” or “3-PPI.” The former response generates second messengers that are responsible for elevating intracellular [Ca2+]i and activating PKC, whereas the latter response is associated with mitogenic effects in cells such as fibroblasts (6). The mechanism by which thrombin activates platelets has been a subject of debate (7). Although thrombin must be proteolytically active to be an effective platelet agonist (8), a role for saturable binding of thrombin to the platelet, in keeping with occupancy of a thrombin receptor, has been suggested (9). The continuous presence of thrombin has been reported to be necessary for the full activation of PLC, based upon inhibitory studies with hirudin, added at various intervals after thrombin (10, 11). Hirudin, in binding thrombin, renders thrombin unavailable to saturable sites on the platelet and interferes with thrombin’s proteolytic function, thereby failing to distinguish between the two potential actions of thrombin. To make such a distinction possible, we have employed in the present studies an inhibitor (DAPA), described by Nesheim et al. (12), which can rapidly inactivate thrombin’s proteolytic activity in situ without, we have confirmed, impairing binding (13).

Recently, the cloning of a functional “thrombin” receptor has permitted a major advance in our understanding of how thrombin can activate platelets (14). Thrombin cleaves a seven-transmembrane domain receptor, and the resulting N-terminal tethered ligand at the cell surface is thought to bind to another region of this receptor. A peptide sequence that duplicates a portion of the tethered ligand, SFLL-RNPNDKYEPF, has been found to cause platelet aggregation and secretion (14). The activation of PLC or 3-PPI accumulation in response to this ligand, however, has not been characterized nor has any possible additional role of thrombin in this setting been examined. Such studies are also presented here.

MATERIALS AND METHODS

Radioisotopes and reagents were obtained as described previously (3, 4). α-Thrombin (1 unit/ml = 10 nm) and DAPA were generous gifts from Dr. K. G. Mann (University of Vermont, Burlington, VT). Hirudin was purchased from Sigma. The Partisphere SAX HPLC column was from Whatman. [32P]PtdIns(3,4)P2 and [32P]PtdIns(3,4,5)P3 were generously contributed by Dr. Peter Downes (University of Dundee, Scotland). PPACK-thrombin and Quick II-thrombin were kindly provided by Dr. P. Tracy (University of Vermont) and Dr. R. A. Henriksen (East Carolina University School of Medicine, NC), respectively. Both thrombins are proteolytically inactive, the former because of chemical alteration of the active site and the latter due to a single amino acid substitution at Gly-558.

1The abbreviations used are: PLC, phospholipase C; PKC, protein kinase C; PtdIns, phosphatidylinositol (locants of phosphates indicated in parentheses); 3-PPI, 3-phosphorylated phosphoinositides; PtdOH, phosphatic acid; FACS, fluorescent-activated cell sorter; DAPA, dabcylarginine-β-(9-ethyl 1,5-pentanedione; PPACK, phenylalanyl-arginylchloromethyl ketone; PDBu, δ-phorbol dibutyrate; SFLL, SFLLRNPNDKYEPF; P47, platelet protein target of PKC; QII, Quick II-thrombin; HPLC, high pressure liquid chromatography; FITC, fluorescein isothiocyanate; GTPγS, guanosine 5’-O-(thiotriphosphate).
within the Arg-binding pocket (15). Both bind with high affinity to platelets,\(^2\) comparable with that of α-thrombin.

**Preparation and Incubation of Platelets—**Human platelets were isolated, labeled with \(^{32}\)P, and as described previously (3, 4). After platelets were incubated with thrombin or hirudin, DAPA or hirudin responded to that observed after 

\[
\text{thrombin} \quad \text{or hirudin}
\]

**RESULTS AND DISCUSSION**

We have drawn two major conclusions regarding the activation of platelet PLC and 3-PPI accumulation by thrombin. 1) Stimulation of both events is dependent upon the sustained presence of proteolytically active thrombin, with no detectable role for saturable thrombin binding, and 2) since a peptide portion of the “thrombin” receptor’s tethered ligand can mimic such activation effects of thrombin completely, without potentiation by thrombin, thrombin’s effects are most probably accounted for by the generation of tethered ligand.

We addressed the hypothesis that, although initiation of one or both aspects of platelet phosphoinositide metabolism by thrombin would be dependent on thrombin’s proteolytic activity, the sustained requirement for thrombin (10) might be dependent upon receptor occupancy, apart from proteolysis. To block thrombin’s proteolytic activity in situ, without impairing thrombin binding, we added DAPA at different intervals after thrombin. To block both thrombin’s binding to and proteolytic interaction with platelets, hirudin was similarly added. DAPA or hirudin, when added mixed with thrombin, completely blocked accumulation of 3-PPI and PtdOH (used as a monitor of PLC activation (17)). Neither DAPA nor hirudin impaired such activation by the thromboxane A\(_2\) mimetic, U46619 (not shown), indicating that platelet metabolism, per se, was not inhibited. Analysis by FACS of fluorescently labeled thrombin binding to platelets indicated that maximal binding was achieved within 3 s of mixing of platelets and thrombin and that addition of DAPA to this mixture did not impair the binding of thrombin to platelets. DAPA bound to and thereby inactivated thrombin maximally in 2–3 s, as did hirudin. This was confirmed by adding platelets to a mixture of DAPA + thrombin or hirudin + thrombin versus adding platelets to DAPA, or hirudin, or buffer, and thrombin in separate droplets. The rate of inactivation by either inhibitor was calculated based upon the initial rate plot of PtdOH formation in response to thrombin, without inhibitors. The amount of PtdOH formed when inhibitors were present, but not premixed, with thrombin corresponded to that observed after 2–3 s exposure to thrombin.
Phosphoinositide Metabolism and Platelet Thrombin Receptor

Table I

Inhibitory effects of DAPA or hirudin when added at various intervals post-thrombin

Platelets were incubated as in Fig. 1. Inhibition after addition of inhibitor at t, was calculated as 100 - (dpm t - dpm t,)/(dpm t, - dpm t,)*100. In the case of *, total incubation period was 60 s rather than 90 s. Hir, hirudin.

| Event inhibited | Inhibitor at 10 s | Inhibitor at 20 s | Inhibitor at 30 s | Inhibitor at 60 s |
|-----------------|------------------|------------------|------------------|------------------|
|                 | DAPA             | Hir              | DAPA             | Hir              |
| Exp. 1          |                  |                  |                  |                  |
| PtdOH           | 86 ± 7           | 86 ± 5           | 79 ± 5           | 72 ± 4           |
| PtdIns(3,4)P₂   | 63 ± 5           | 66 ± 5           | 48 ± 7           | 50 ± 3           |
| Exp. 2*         |                  |                  |                  |                  |
| PtdOH           | 72               | 70               | 58               | 62               |
| PtdIns(3,4)P₂   | 27               | 25               | 15               | 16               |
| Exp. 3          |                  |                  |                  |                  |
| PtdOH           | 76 ± 2           | 71 ± 1           | 65 ± 1           | 59 ± 10          |
|                 |                  |                  | 50 ± 2           | 51 ± 6           |
|                 |                  |                  |                  | 31 ± 3           |
|                 |                  |                  |                  | 30 ± 7           |

The data are the average ± range of duplicates and are expressed as a percent of basal values (=100). P47 was included as a monitor of PKC activation.

Fig. 2: Effects of proteolytically inactive thrombin on 3-PPI accumulation. Platelets labeled as in Fig. 1 were incubated for 90 s with buffer, QII thrombin (200 nM), PPACK-thrombin (250 nM), thrombin (2 units/ml, 20 nM), PDBu (200 nM) + A23187 (1 μM), PDBu + A23187 + QII thrombin, or PDBu + A23187 + PPACK-thrombin (THR). Incubations were terminated and phosphoinositides and P47 protein resolved and ³P quantitated as described. Results are the average ± range of duplicates and are expressed as a percent of basal values (=100). P47 was included as a monitor of PKC activation.

Fig. 3: Effects of α-thrombin, SFLL, or α-thrombin + SFLL on accumulations of PtdIns(3,4)P₂ and PtdOH in human platelets with time. Human platelets, as in Fig. 1, were incubated with 2 units/ml α-thrombin or 400 μM SFLL for various times. The data are representative of two experiments and are expressed as a percent of agonist-free control values (=100) with error ranges encompassed by symbols. ○, α-thrombin; □, SFLL; △, both; broken line, PtdOH; solid line, PtdIns(3,4)P₂.

As can be seen in Fig. 1 and Table I, there was no significant difference between the effects of DAPA and hirudin on the inhibition of PtdOH or 3-PPI (as represented by PtdIns(3,4)P₂) accumulation. Sustained receptor occupancy (DAPA experiment) thus had no potentiating effect independent of proteolysis. The crucial inhibitory event was clearly blockage of thrombin’s proteolytic function, both initially and after 10-, 20-, 30-, or 60-s exposures to thrombin (Table I). Any role for saturable binding of thrombin to the platelet in modulating phosphoinositide metabolism is thus most likely explained in terms of binding to an enzymatic substrate as opposed to a receptor coupled to an intracellular effector. PtdOH accumulation (PLC activation) was consistently more impaired by each inhibitor than was accumulation of 3-PPI (Table I). Further, addition of excess PPACK-thrombin, prior to thrombin addition, affected neither PtdOH nor 3-PPI (not shown). This finding indicates that proteolytically active thrombin need not even bind to all saturable sites on the platelet to achieve its agonist effects on phosphoinositide metabolism.

Since we have demonstrated recently that maximal accumulation of 3-PPI is dependent upon PKC activation and that PKC activation is necessary but not sufficient for the full 3-PPI response, we wondered what the additional requisite factor might be, apart from PLC-derived second messengers. We therefore added agents that would increase PKC activity (PDBu), monitored by P47 phosphorylation, and elevate cytosolic [Ca²⁺] (A23187) and examined the effect of saturating amounts of two different proteolytically inactive thrombins (PPACK-thrombin and TQII) upon accumulations of 3-PPI. This was compared with the effects of proteolytically active thrombin, which stimulated PKC to an extent similar to that achieved with PDBu. The results summarized in Fig. 2 illustrate that the missing factor is clearly not saturable thrombin, consistent with the findings in Fig. 1. Neither form of proteolytically active thrombin altered the 3-PPI response, even when PLC-dependent second messenger signals were provided independently of PLC activation. Thus, results of both Figs. 1 and 2 point exclusively to the role of proteolysis in implementing thrombin’s effects on both aspects of phosphoinositide metabolism.

Finally, we turned to the question of whether thrombin’s essential proteolytic effects could be accounted for by creation of tethered ligand. Fig. 3 shows that, in response to either thrombin (2 units/ml) or SFLL (400 μM), PtdIns(3,4)P₂ increased in a manner sustained for at least 60 s. PtdIns(3,4,5)P₃ increased rapidly to a maximum level within 30 s (not shown). In contrast, PtdOH accumulated more rapidly in response to SFLL than to thrombin but leveled off after 20 s, when SFLL was the agonist, whereas PtdOH continued to increase in
response to thrombin up to (Fig. 3) and beyond (Fig. 1) 60 s. This finding for PtdOH can be explained by the continued generation of tethered ligand by thrombin, eventually achieving a level exceeding that mimicked by SFLL, and by the greater requirement of PLC versus 3-PPI activation for agonist concentration (see below). It is evident (Fig. 4) that the 3-PPI response is more sensitive to either thrombin or SFLL than is PLC activation. Concentrations of agonist producing a half-maximal 3-PPI response were 0.075 units/ml thrombin and 25 μM SFLL; those leading to half-maximal PtdOH were 0.5 units/ml thrombin and 300 μM SFLL. This difference most likely accounts for the lesser sensitivity of 3-PPI accumulation (versus PLC activation) to the addition of DAPA or hirudin post-thrombin (Table I). Significantly, when SFLL and thrombin were added simultaneously to platelets, stimulation of 3-PPI was not additive but was only marginally greater than when either was added alone (Fig. 3). Maximum platelet enzymatic capacity had not been reached, however, since we have shown that the potent G protein-directed agonist, GTPγS, can achieve a stimulation that greatly exceeds that for thrombin (3, 4). Thus, binding of thrombin or additional proteolytic targets for thrombin apart from tethered ligand generation contributed nothing more in promoting PLC activation and 3-PPI accumulation.

Making a comparison between thrombin/tethered ligand and SFLL in terms of stoichiometry is complicated by three factors. 1) Tethered ligand, as a function of thrombin’s sustained proteolytic activity on platelets, is apparently generated over the time course (at least 60 s) of platelet incubations with thrombin, whereas SFLL is present maximally at the outset; 2) SFLL may not be the optimal size or in optimal tethered ligand is generated near its receptor, and therefore its efficiency of binding or binding rate is most likely much greater than that of SFLL. Indeed, were all of 400 SFLL, stoichiometries cannot be compared. However, lack of potentiation of SFLL’s effects by thrombin would indicate that thrombin is most likely not acting either proteolytically at additional sites distant from the cleavage site which generates the tethered peptide or as a direct ligand in order to exert its effects on phosphoinositide metabolism. These findings support the conclusion that the majority of thrombin’s effects on phosphoinositide metabolism relate to the generation of tethered ligand.

We have shown that a tethered peptide analogue activates platelet PLC and 3-PPI accumulation in a manner unmodified by proteolytically active thrombin and can achieve maximal effects similar to those of thrombin. It is therefore evident that future work directed toward an understanding of how thrombin-activated PKC, G protein(s), and other factors regulate PLC and 3-PPI accumulation should focus on the receptor target (14) of the tethered ligand. Our data would also indicate that it is primarily receptor occupancy by tethered ligand that, in addition to activating PLC/PKC, plays a direct role in promoting 3-PPI accumulation.

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