Effects of separate proteolytic and high-affinity binding activities of human thrombin on rapid platelet activation

A quenched-flow study

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We have used a general quenched-flow approach to study platelet function as early as 0.3 s after stimulation with three types of human thrombin: α -thrombin, γ -thrombin, which is proteolytically active but does not bind to the high-affinity sites, and di-isopropyl fluorophosphate-derivatized (DIP)- α -thrombin, an active site-inhibited analogue that does bind to the high-affinity site. Large doses of γ -thrombin evoked moderate aggregation and serotonin release, but minimal phosphorylation of the 20 and 47 kDa proteins. The initial (1.5–3.0 s) increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) indicated by Indo-1 was also diminished, but by 5 s was nearly as high (1.0 μ M) as with α -thrombin. A large dose of DIP- α -thrombin, on the other hand, induced minimal aggregation, serotonin secretion and $[Ca^{2+}]_i$ response within 6 s. There was, however, a transient dephosphorylation of the 20 kDa protein. When combined, γ - and DIP- α -thrombin were approximately additive in their ability to induce aggregation and serotonin secretion, but strongly synergistic in phosphorylating the 20 and 47 kDa proteins. The $[Ca^{2+}]_i$ increase was not, however, enhanced over that induced by γ -thrombin alone. These results demonstrate that phosphorylation of either the 20 or 47 kDa proteins is not correlated with $[Ca^{2+}]_i$ dynamics and is neither required nor directly involved in platelet aggregation and secretion induced by thrombin. The high-affinity binding activity of thrombin is not necessary for rapid platelet Ca^{2+} influx, aggregation and serotonin release within the first critical seconds of activation.

INTRODUCTION

The serine protease α -thrombin is a potent platelet activating agent as well as an important physiological mediator in haemostasis. Platelet activation results in rapid morphological changes, aggregation, granule secretion, ion fluxes [1–3], increased turnover of phosphatidylinositol [4,5], phosphorylation of specific proteins [6] and binding of fibrinogen to surface receptors [7]. Two types of interactions of thrombin with blood platelets have been identified: binding of α -thrombin to specific sites of varying affinity on the platelet surface [8] and hydrolysis of platelet membrane glycoprotein V [9]. Recently, studies with thrombin analogues have suggested that occupancy of a high-affinity binding site for thrombin is required for platelet activation [8]. However, thrombin must be both proteolytically active and bind to a high-affinity receptor for maximal platelet activation [10]. The molecular mechanisms through which thrombin induces aggregation and secretion remain unclear, partly due to the rapidity with which platelet responses occur. Morphological changes and aggregation are complete within seconds of an inducing stimulus [11,12].

The quenched-flow approach [11] has previously been used to study morphological changes [12], secretion [13], protein phosphorylation [14,15] and aggregation [11] within the time scale required for efficient haemostasis,

which is estimated to be about 0.1 s [16]. Both aggregation, as detected by single-particle counting [17], and phosphorylation of the 20 kDa (myosin light chain; MLC) and 47 kDa proteins are significant within 0.3 s and reach a plateau by 3–5 s; the thrombin sensitivity of aggregation and 20 kDa protein phosphorylation is approximately twice that of 47 kDa protein phosphorylation [14]. Chelation of extracellular Ca^{2+} significantly inhibits aggregation and MLC phosphorylation (≥ 0.6 s), but not 47 kDa-protein phosphorylation. We recently modified the quenched-flow approach to measure the cytosolic free calcium concentration $[Ca^{2+}]_i$ under physiological blood-flow conditions [18]. Thrombin causes a sustained $[Ca^{2+}]_i$ increase that is significant within 0.5 s, with the second phase (> 2 s) being partially dependent upon extracellular Ca^{2+} .

In the present study we report the effects of α -thrombin, partially proteolysed γ -thrombin [19,20], active-site inhibited di-isopropyl fluorophosphate-derivatized (DIP)- α -thrombin [19,21], or a combination of γ - and DIP- α -thrombin on serotonin secretion, $[Ca^{2+}]_i$ dynamics, protein phosphorylation and aggregation during the first 6 s of platelet activation. γ -Thrombin is proteolytically active but does not bind to the platelet's high-affinity sites and has very low coagulant activity [9], whereas DIP- α -thrombin is not proteolytically active but does bind to the high-affinity sites [10,22]. The aim of these

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free calcium concentration; DIP- α -thrombin, di-isopropyl fluorophosphate-derivatized α -thrombin; MEM, minimum essential medium; MLC, myosin light chain; PRE, platelet-rich Eagle's MEM; PPE, platelet-poor Eagle's MEM; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

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studies was to understand better the mechanisms behind the rapid activation of platelet function by human α -thrombin. Some of the results of this study have been communicated in preliminary form [23].

MATERIALS AND METHODS

Materials

Highly purified human α -, γ - and DIP- α -thrombin were obtained from Dr. John W. Fenton, II, Division of Laboratories and Research, New York State Dept. of Health, Albany, NY, U.S.A. and were stored at -70°C ; the γ -thrombin contains 0% α , $\leq 0.4\%$ β , and $\geq 99.6\%$ γ as assayed by Dr. Fenton. The sources for other supplies were Fisher Scientific Co. (Scinti Verse E liquid scintillation cocktail); Gibco, Grand Island, NY, U.S.A. (minimum essential medium; MEM); Molecular Probes, Eugene, OR, U.S.A. (Indo-1/AM); DuPont NEN Medical Products, North Billerica, MA, U.S.A. (^{32}P) and Sigma Chemical Co., St. Louis, MO, U.S.A. (prostacyclin, indomethacin, essentially fatty acid-free albumin, Hepes, hirudin, human fibrinogen and grade VII apyrase). All solvents and other chemicals were reagent grade or better.

Human subjects

Investigations were performed after approval by the local human investigation committee and in accord with an assurance filed with and approved by the Dept. of Health and Human Services.

Preparation of human platelets

Platelet-rich plasma (PRP) was isolated from human venous forearm blood collected into acid/citrate/dextrose as previously described [11].

Platelet $[\text{Ca}^{2+}]_i$ dynamics: continuous-flow studies

Platelets were loaded by incubating PRP containing 8 units of apyrase/ml, $0.3\text{ }\mu\text{g}$ of prostacyclin/ml, $1\text{ }\mu\text{g}$ of indomethacin/ml and additional acid/citrate/dextrose (1:20 initial volume), with $2.5\text{ }\mu\text{M}$ -Indo-1/AM [24] for 30 min at 37°C . The platelets were then washed and suspended in 0.5 vol. of Eagle's MEM buffer (pH 7.2) as previously described [18] except that 0.5 mg of human fibrinogen/ml was added to the PRE (platelet-rich Eagle's MEM). This suspension was gassed with a CO_2 /air (1:19) mixture to preserve platelet function [25]. The design of a flow-through fluorescence microcuvette and details of our continuous-flow procedures have been described fully [18]. Data are recorded from a T-format spectrofluorimeter (model 4800; SLM Instruments, Urbana, IL, U.S.A.) as a corrected ratio of the simultaneously monitored 400 nm and 480 nm Indo-1 emission signals, which is proportional to $[\text{Ca}^{2+}]_i$. The derivation of the corresponding $[\text{Ca}^{2+}]_i$ assignments has been described [18].

Platelet aggregation and serotonin secretion: quenched-flow studies

Platelets were labelled by incubating PRP with $0.2\text{ }\mu\text{Ci}$ of $[2\text{-}^{14}\text{C}]$ serotonin/ml for 20 min at 37°C ; the above-listed quantities of apyrase, prostacyclin, indomethacin and acid/citrate/dextrose were added immediately following this incubation. The platelets were then washed as described for $[\text{Ca}^{2+}]_i$ determinations, except that an equal volume of Eagle's MEM was used for the final

suspension. Aliquots of PRE (statics) were used to provide values for platelets not subjected to pumping through the quenched-flow system.

Prior to each quenched-flow 'run', an aliquot of PRE incubated with $4.2\text{ }\mu\text{M}$ -imipramine for 5 min at 37°C to prevent re-uptake of serotonin during the 'run' [13] was placed in one syringe of the quenched-flow apparatus [11,13]. The second and third syringes were filled with agonist and quenching agent [1% (v/v) glutaraldehyde] respectively. The principle behind this system is to pump platelets and agonist through narrow-bore tubing (0.2–0.3 mm internal diameter) and then quench the reaction at the outlet. The reactions were carried out at 37°C and the speed of quenching is estimated to be 0.1 s [14]. Samples of the quenched effluent were collected for each reaction time.

The extent of platelet aggregation in each sample was evaluated using single-particle counting [17] as previously described [11,14]. Aggregation is expressed as the percentage of single platelets remaining at each reaction time relative to the static value.

For the determination of serotonin release, each quenched sample was centrifuged for 5 min at 6000 g; $50\text{ }\mu\text{l}$ aliquots of the resulting PPE (platelet-poor Eagle's MEM) were mixed with liquid scintillation cocktail and counted for ^{14}C . The serotonin release is expressed as the percentage of ^{14}C in each sample relative to the static PRE and PPE.

Platelet protein phosphorylation: quenched-flow studies

Platelets were incubated with $^{32}\text{P}_i$, washed and suspended in Eagle's MEM as previously described [15]. The quenched-flow procedure was the same as for aggregation and serotonin secretion, except that 3 M-perchloric acid was used as the quenching agent and imipramine was not added to the PRE. (Aggregation also was followed using glutaraldehyde with this series of experiments; results not shown.) Protein phosphorylation was assessed as previously described [14,26], except that the autoradiographs were integrated using a micro-computer (model 6300; AT&T, Morristown, NJ, U.S.A.) that accepts digitized images from a video camera (series 66; DAGE-MTI, Inc., Michigan City, IN, U.S.A.) via an image capture board (PC Vision Frame Grabber, Woburn, MA, U.S.A.); custom software was written by the Computer Technology Center (University of Virginia). Autoradiographic data were stored and analysed with a spreadsheet program (LOTUS 1-2-3; Lotus Development Corp., Cambridge, MA, U.S.A.).

Statistical analysis

Student's *t*-test for either paired or unpaired samples, as appropriate, was used to examine statistical differences due to reaction time or inducing agent. Values are presented as means from different PRP preparations $\pm$ S.E.M.

RESULTS

Platelet aggregation kinetics

The kinetics of aggregation measured by single-particle counting [17] reflect very early events in platelet function ($< 5\text{ s}$) [11–15,18]. Pumping the platelets through the quenched-flow system in the absence of agonist (control) did not cause any significant shear-induced aggregation.

Fig. 1. Kinetics of platelet aggregation induced by human α -thrombin, γ -thrombin and DIP- α -thrombin

Aggregation was evaluated using single-particle counting and is expressed as the percentage of single platelets remaining at each reaction time relative to the static value. The washed platelets were reacted with agonist in the quenched-flow apparatus as described in the Materials and methods section. Nine different PRE preparations were studied, with 8–9 measurements at each reaction time and condition. The data are expressed as means $\pm$ S.E.M. Concentrations of agonists in the reaction loop were: 80 nM- α -thrombin ($\square$), 500 nM- γ -thrombin ($\blacktriangle$), 500 nM-DIP- α -thrombin ($\blacktriangledown$) and a combination of 500 nM each of γ - and DIP- α -thrombin ($\blacklozenge$).

On the other hand, 80 nM (8 units/ml) human α -thrombin induced rapid aggregation that was significant ($P < 0.01$) by 0.6 s (results not shown); only 72.4% and 41.7% of single platelets remained at 1.5 and 5 s respectively (Fig. 1). This was comparable to that previously found with bovine thrombin [14]. This concentration of thrombin is approximately that achieved during the clotting of whole blood [27]. Whereas 52 nM of either γ -thrombin or DIP- α -thrombin did not induce significant aggregation (results not shown), higher doses (500 nM) did cause aggregation. Like 80 nM- α -thrombin, the aggregation induced by 500 nM- γ -thrombin was significant by 0.6 s ($P < 0.01$, results not shown) but then slowed; 89.2% and 59.2% of single platelets remained at 1.5 and 5 s, respectively (Fig. 1). A concentration of 500 nM-DIP- α -thrombin caused less aggregation; 94.3 and 79.3% single platelets remained at 1.5 and 5 s respectively (Fig. 1). We then studied the effects of a combination of 500 nM- γ -thrombin and 500 nM-DIP- α -thrombin. This high-dose combination induced an aggregation response nearly identical to that for 80 nM- α -thrombin (Fig. 1); 75.5 and 46.6% of single platelets remained at 1.5 and 5 s respectively; there was no significant difference compared with 80 nM- α -thrombin. Therefore, for the remainder of our studies, we decided to investigate several biochemical effects of the high concentrations of γ and DIP- α -thrombins relative to the lower physiological levels of α -thrombin.

Cytosolic calcium kinetics

The data presented in Fig. 2 illustrate the effects of the different thrombins on the rate and extent of early (< 6 s) changes in platelet $[\text{Ca}^{2+}]_i$ as indicated by Indo-1

Fig. 2. Rapid $[\text{Ca}^{2+}]_i$ dynamics in platelets stimulated by α -thrombin, γ -thrombin and DIP- α -thrombin

Washed, Indo-1-loaded platelets were monitored in the flow-through fluorescence microcuvette [18]. Values are shown as means $\pm$ S.E.M. of the Indo-1 fluorescence ratio, with corresponding $[\text{Ca}^{2+}]_i$ assignments on the right ordinate. The static control value was previously determined [18]. Six different PRE preparations were studied, with 4–8 different measurements for each reaction time and condition. Concentrations of agonists in the reaction loop were: saline control ($\circ$), 80 nM- α -thrombin ($\square$), 500 nM- γ -thrombin ($\blacktriangle$), 500 nM-DIP- α -thrombin ($\blacktriangledown$) and a combination of 500 nM each of γ - and DIP- α -thrombin ($\blacklozenge$).

fluorescence. Control experiments revealed that the shear stresses of continuously pumping platelets through the reaction tube only slightly increased the $[\text{Ca}^{2+}]_i$ to 112 nM on average, from a static level of 100 nM [18]. As with aggregation, the most potent agonist tested was α -thrombin; it increased $[\text{Ca}^{2+}]_i$ to 0.19, 0.74 and 1.95 μM at 1.0, 2.0 and 5.4 s respectively (Fig. 2). We have previously shown that the $[\text{Ca}^{2+}]_i$ increase induced by either ADP (10 μM) or bovine thrombin (52 nM) is significantly above control levels ($P < 0.001$) within 0.5 s [18]. Whereas DIP- α -thrombin did not rapidly increase $[\text{Ca}^{2+}]_i$ (0.20 μM at 5.4 s), γ -thrombin resulted in 1.05 μM - $[\text{Ca}^{2+}]_i$ at 5.4 s (Fig. 2). The γ -thrombin was initially slower than the α -thrombin at increasing $[\text{Ca}^{2+}]_i$, resulting in only 0.25 μM at 2.0 s. The combination of γ -thrombin and DIP- α -thrombin was clearly not synergistic for the $[\text{Ca}^{2+}]_i$ response; initially the combination acted nearly identically to γ -thrombin, but after 3.0 s the $[\text{Ca}^{2+}]_i$ rise (0.61 μM at 5.4 s) was lower. The $[\text{Ca}^{2+}]_i$ increase induced by the combination was significantly less than for α -thrombin at 1.4, 2.0 and 5.4 s ($P < 0.05$). Although DIP- α -thrombin barely increased $[\text{Ca}^{2+}]_i$ before 5 s, a peak near 1.0 μM occurred much later (Fig. 3).

Serotonin secretion kinetics

The time courses of [^{14}C]serotonin secretion (Fig. 4) from platelets paralleled the aggregation response for each agonist. Control 'runs' resulted in an average release of 5.4% of platelet serotonin at all reaction times. α -Thrombin released 22.4% serotonin by 1.5 s and 45.2% by 5.0 s. The combination of γ - and DIP- α -thrombin was less effective than α -thrombin, although the difference was only significant during the first 3 s ($P < 0.05$). The release induced by γ -thrombin, DIP- α -thrombin or the combination of the two was 11.1, 6.0 and 12.1% at 1.5 s and 25.1, 12.5 and 32.3% at 5.0 s respectively.

Fig. 3. Kinetics of DIP- α -thrombin-induced $[Ca^{2+}]_i$ changes in human platelets

The fluorescence of washed, Indo-1-loaded platelets was monitored as described in the Materials and methods section and the legend to Fig. 2, except the platelets were not continuously pumped during these 'runs'. The length of the reaction tube and the syringe pump speed were set for a reaction time of 1.0 s. The corrected fluorescence ratio was acquired at either 1 s or 5 s intervals after the pump was stopped. These data, unlike all the rest that we report, were not obtained from platelets reacted under near-rheological conditions. The $\blacktriangledown$ symbols represent the results from one experiment (1 s intervals) with 500 nM-DIP- α -thrombin. The curve is drawn through the means $\pm$ S.E.M. of eight similar 'runs' (5 s intervals).

Phosphorylation of the 20 kDa protein

As for other platelet functions, α -thrombin was also the strongest inducer of phosphorylation of the 20 kDa protein (MLC); this was 36, 62 and 164 % above control values at 0.3, 1.0 and 5.0 s respectively (Fig. 5a). Neither 52 nM- γ -thrombin nor 52 nM-DIP- α -thrombin induced significant phosphorylation (results not shown). Whereas a larger dose of γ -thrombin did not induce a significant

Fig. 4. Kinetics of $[^{14}C]$ serotonin release from platelets stimulated by α -thrombin, γ -thrombin and DIP- α -thrombin

The PRE preparations used and reaction conditions employed were the same as for Fig. 1. Serotonin released into the extracellular fluid is expressed as a percentage of ^{14}C relative to the static PRE and PPE. Concentrations of agonists in the reaction loop were: saline control ($\circ$), 80 nM- α -thrombin ($\square$), 500 nM- γ -thrombin ($\blacktriangle$), 500 nM-DIP- α -thrombin ($\blacktriangledown$) and a combination of 500 nM each of γ - and DIP- α -thrombin ($\blacklozenge$).

Fig. 5. Kinetics of phosphorylation of two platelet proteins: (a) 20 kDa, (b) 47 kDa

Washed, ^{32}P -loaded platelets were reacted and quenched in the quenched-flow apparatus and protein phosphorylation assessed as described in the Materials and methods section. The data are expressed as means $\pm$ S.E.M. of twelve experiments with 52 nM- α -thrombin and four experiments with the other agonists. Concentrations of agonists in the reaction loop were: 52 nM- α -thrombin ($\square$), 52 nM- γ -thrombin ($\blacktriangle$), 52 nM-DIP- α -thrombin ($\blacktriangledown$) and a combination of 52 nM each of γ - and DIP- α -thrombin ($\blacklozenge$).

phosphorylation until 5 s (26 % above control, $P < 0.05$), the DIP- α -thrombin actually caused an early and significant transient decrease in phosphorylation of MLC (29 % below control at 1.0 s, $P < 0.05$) followed by a return to control levels by 3.0 s. The combination of γ - and DIP- α -thrombin acted more like α -thrombin than either γ - or DIP- α -thrombin alone. Although the combination was initially slower than α -thrombin at inducing phosphorylation (33 % above control at 1.0 s), the difference between the two at 5.0 s was not significant.

Phosphorylation of the 47 kDa protein

The phosphorylation of the 47 kDa protein (apparent molecular mass 44 kDa on our gels) generally lagged behind that of 20 kDa protein (Fig. 5b). DIP- α -thrombin had no effect on the protein's phosphorylation, and that induced by γ -thrombin was not significant until 5.0 s (20 % above control, $P < 0.05$). α -Thrombin induced a strong phosphorylation of the 47 kDa protein to 13, 47 and 118 % above control at 0.3, 1.0 and 5.0 s respectively; it was significantly above control at 1.0 s ($P < 0.05$). Although the phosphorylation induced by a combination

of γ - and DIP- α -thrombin [5 % below control at 0.3 s, 14 % above control at 1.0 s ($P < 0.05$)] was initially slower than with α -thrombin alone, it actually surpassed the α -thrombin at 5.0 s (152 % above control).

DISCUSSION

Platelet activation by thrombin is very rapid as revealed by our new quenched- and continuous-flow methodologies under rheological conditions close to those *in vivo* [11,28]. Aggregation, morphological changes, secretion, protein phosphorylation and increased $[Ca^{2+}]_i$ are essentially complete within 5 s [11–15,18]. In the present study we have investigated how the separate high-affinity binding and proteolytic components of thrombin are involved in these early initiation phases of platelet function.

α -Thrombin

Maximal platelet activation is evoked by α -thrombin, which is both proteolytically active and binds to a high-affinity receptor [10]. We have shown that purified human α -thrombin induces rapid and significant aggregation, $[Ca^{2+}]_i$ increase, serotonin secretion and protein phosphorylation within the first seconds of platelet activation. These results are similar to those previously reported for bovine thrombin [13,14,18].

Proteolytically active thrombin

γ -Thrombin, a partially proteolysed product of α -thrombin, does not bind to the high-affinity site but retains proteolytic activity [9]. Surprisingly, large doses caused moderate aggregation (Fig. 1) and secretion of serotonin (Fig. 4), but these occurred in the absence of the phosphorylation of the 20 and 47 kDa proteins (Fig. 5).

The initial (1.5–3.0 s) increase in $[Ca^{2+}]_i$ elicited by γ -thrombin was slower than that caused by α -thrombin (Fig. 2). Our previous work with bovine thrombin demonstrated that the initial major rise in $[Ca^{2+}]_i$ does not involve extracellular Ca^{2+} , whereas both internal and external sources of Ca^{2+} contribute to a second phase beginning near 2 s [18]. This suggests that rapid release of internal Ca^{2+} stores by γ -thrombin either does not occur or is delayed; this may partially explain the low level of MLC phosphorylation during the first 6 s. The relatively slow but major γ -thrombin-induced increase in $[Ca^{2+}]_i$ (1.05 μ M at 5.4 s) suggests, however, that the high-affinity thrombin site is not required for activation of Ca^{2+} influx.

The mechanisms leading to thrombin-induced phospholipase C activation remain unclear. It has recently been shown that γ -thrombin is a more potent stimulator of membrane-associated $PtdIns(4,5)P_2$ phospholipase C *in vitro* than is α -thrombin [29]. In intact platelets, however, γ -thrombin is less potent and slower at inducing hydrolysis of phosphoinositides [5], thus explaining the slower $[Ca^{2+}]_i$ response we observed. Although several platelet reactions are sluggish as compared with α -thrombin, our results indicated that the high-affinity binding activity of thrombin was not necessary for rapid platelet aggregation, serotonin secretion or $[Ca^{2+}]_i$ influx.

High-affinity-binding thrombin

DIP- α -thrombin is not proteolytically active but does bind to the high-affinity site [10,22]. Large doses of DIP-

α -thrombin evoked only minimal responses during the first seconds of platelet activation; thus aggregation (Fig. 1), serotonin secretion (Fig. 4) and Ca^{2+} fluxes (Fig. 2) were only slightly activated by 6 s. DIP- α -thrombin did, however, cause a slower, sustained $[Ca^{2+}]_i$ increase that reached 1.0 μ M by 15 s (Fig. 3). The large dose of DIP- α -thrombin induced a rapid, transient dephosphorylation of MLC (Fig. 5a), although there was no change in the degree of phosphorylation of the 47 kDa protein (Fig. 5b). These results are compatible with the recent report that DIP- α -thrombin does not activate phospholipase C [29]. One proposed model suggests that the high-affinity binding activity of thrombin is responsible for the inhibition of adenylyl cyclase and activation of phospholipase A_2 , but not the activation of phospholipase C [30]. Since DIP- α -thrombin did not cause phosphorylation of the 47 kDa protein, the small extent of aggregation at 5 s may be due to the secondary actions of arachidonic acid.

Combined high-affinity and proteolytic activities of thrombin

The two thrombins were approximately additive in their ability to induce the functional responses of aggregation (Fig. 1) and serotonin secretion (Fig. 4); they were strongly synergistic in causing rapid phosphorylation of the 20 and 47 kDa proteins (Fig. 5). These responses approached those elicited by α -thrombin. However, the $[Ca^{2+}]_i$ response induced by the combination was the same as for γ -thrombin alone, except after 5 s (Fig. 2). Thus, substantial platelet activation occurred in the absence of the usual large and rapid (< 2 s) increase in $[Ca^{2+}]_i$ derived from intracellular sources [18], as indicated by Indo-1 fluorescence. The lack of correlation between $[Ca^{2+}]_i$ and protein phosphorylation is particularly intriguing, suggesting that calcium is not a major 'signal' for protein phosphorylation. These results support our recent suggestion of a calcium-independent protein kinase acting on the 20 kDa protein [31], or the existence of a calcium pool not sensed by Indo-1. Additionally, it is now known that different peptides on the 20 kDa protein can be phosphorylated by a calcium-dependent protein kinase and protein kinase C [32].

Concluding remarks

The results of the present study demonstrate that, under rheological conditions similar to those *in vivo* [11,28], phosphorylation of the platelet MLC and 47 kDa proteins by thrombin activation, like that by ADP [31], was not correlated with $[Ca^{2+}]_i$ dynamics and was neither required nor directly involved in platelet aggregation and secretion. This is compatible with a recent report of one isoenzyme of protein kinase C in human platelets that is not sensitive to Ca^{2+} [33]. In addition, we demonstrate that the high-affinity binding of thrombin is not necessary for aggregation, secretion and Ca^{2+} influx.

It might be suggested that a small contamination of γ - and DIP- α -thrombins by active α -thrombin could explain the aggregation we observed at high concentrations of γ - or DIP- α -thrombin. However, these preparations contained no α -thrombin as assayed by Dr. Fenton and, in addition, caused no significant phosphorylation of either the 20 or 47 kDa proteins. In sharp contrast, γ -thrombin induced a major $[Ca^{2+}]_i$ increase (1 μ M by 5 s) during which time neither 20 nor 47 kDa protein phosphorylation was noted. The released calcium thus is unable to

activate MLC kinase, which suggests strongly that another factor is involved in causing the rapid and major phosphorylation seen with either α -thrombin or the combination of γ - and DIP- α -thrombin.

It has recently been proposed by Harmon & Jamieson [34] that a 'moderate-affinity platelet binding site for α -thrombin is a receptor, occupancy of which is required for platelet activation in the absence of the high-affinity receptor.' The high-affinity thrombin receptor was removed by treating platelets with protease, and although these modified platelets are less sensitive, they are fully responsive to α -thrombin, as measured by serotonin secretion. Furthermore, blocked thrombin analogues similar to γ - and DIP- α -thrombin compete with α -thrombin in binding to the moderate-affinity site. It is not clear how this moderate-affinity receptor may be involved in normal platelet activation. Some of the actions of γ -thrombin that we observed could be mediated by this moderate-affinity site. Additional understanding of the actions of the various thrombin receptors may come from using the quenched-flow approach to study the rapid reactions of modified platelets, such as those lacking the high-affinity receptor [34].

The finding of little correlation between $[Ca^{2+}]_i$, the phosphorylation of the 20 and 47 kDa proteins and platelet function is similar to our recent observations on adrenaline-potentiated aggregation induced by ADP [31]. In that case, we observed that adrenaline actually inhibited phosphorylation, even though aggregation and $[Ca^{2+}]_i$ were increased. Consequently, the signal transduction mechanisms leading to functional platelet activation by thrombin, or by ADP and adrenaline, are more complex than previously thought.

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