Pergamon

Biochemical Pharmacology, Vol. 49, No. 4, pp. 519–528, 1995.
Copyright © 1995 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0006-2952/95 $9.50 + 0.00

0006-2952(94)00473-0

INHIBITION OF THROMBIN AND SFLLR-PEPTIDE STIMULATION OF PLATELET AGGREGATION, PHOSPHOLIPASE A_2 AND Na^+/H^+ EXCHANGE BY A THROMBIN RECEPTOR ANTAGONIST

STEVEN M. SEILER,*† MARIANNE PELUSO,* INGE M. MICHEL,* HAROLD GOLDENBERG,* JOHN W. FENTON II,‡ DOUGLAS RIEXINGER§ and SESHA NATARAJAN§

Departments of *Cardiovascular Biochemistry and §Chemistry, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543; and ‡NY State Department of Health, Albany, NY 12201, U.S.A.

(*Received* 25 *February* 1994; *accepted* 2 *September* 1994)

Abstract—A thrombin receptor has been described that is activated by thrombin cleavage generating a new N-terminus. The newly exposed SFLLR-containing "tethered-ligand" then activates the receptor. In these studies, we used 3-mercapto-propionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-amide (Mpa-peptide) as a thrombin receptor antagonist. This compound was capable of preventing both thrombin- and SFLLR-peptide-induced platelet aggregation with little effect on collagen-induced platelet aggregation. It also prevented thrombin- and SFLLRNP-induced calcium mobilization with little effect on thromboxane receptor-activated platelet Ca^{2+} mobilization. Platelet membrane GTPase could be activated by peptides that activated the thrombin receptor, and the thrombin receptor antagonist also prevented receptor-stimulated GTPase activity. Platelet phospholipase A_2 (PLA_2) activity (measured as the release of radiolabeled arachidonic acid) and Na^+/H^+ exchange activation were stimulated by α-thrombin and by SFLLR-containing peptides. Activation of both processes with low concentrations of thrombin required thrombin's anion-binding exosite, as they were not activated by similar concentrations of γ-thrombin, and the α- and ζ- thrombin activation was blocked by peptides mimicking the C-terminal region of hirudin. Stimulation of PLA_2 and Na^+/H^+ exchange by both thrombin and SFLLR-containing peptides was inhibited by the thrombin receptor antagonist Mpa-peptide. These results support the hypothesis that thrombin stimulation of PLA_2 activity and Na^+/H^+ exchange occurs via activation of the thrombin tethered-ligand receptor. Moreover, these data are consistent with the tethered-ligand receptor mediating most actions elicited by low concentrations of α-thrombin involved in human platelet activation.

Key words: thrombin receptor; thrombin; platelet; phospholipase A_2; Na^+/H^+ exchange; thrombin receptor antagonist

A thrombin receptor, discovered by direct expression cloning of mRNA, has been identified from human HEL and Dami cells [1], hamster fibroblasts [2], and rat aortic smooth muscle cells [3]. This receptor is a member of the seven-transmembrane spanning G-protein coupled receptor superfamily [1]. Thrombin binds to this receptor utilizing its anion-binding exosite. A portion of the receptor sequence resembles the carboxy-terminus of hirudin, and thrombin inhibitors that bind to the anion-binding exosite of α-thrombin prevent cleavage of this receptor [1, 4, 5]. Thrombin activates the receptor by cleaving a single peptide bond. The resulting new N-terminus serves as a "tethered-ligand," which activates the receptor [1, 4]. A peptide whose sequence is identical to the new N-terminus (SFLLR) can also activate this receptor, whereas related peptides (acetylated-SFLLR- and FSLLR-containing peptides) could not. More recent studies have demonstrated that peptides truncated to five amino acids (SFLLR) retain receptor-activating activity [6–9].

The involvement of the thrombin tethered-ligand receptor has been implicated by the ability of SFLLR-containing peptides to mimic thrombin cellular responses. These peptides cause platelets to undergo shape change, aggregation, and degranulation [1]. Activation of the platelet thrombin receptor causes activation of phospholipase C [10], intracellular Ca^{2+} mobilization [1], and inhibition of platelet adenylyl cyclase [11]. Blocking antibody studies [12, 13] and studies with SFLLR-peptide-induced desensitization [14] have suggested that the peptide-activated receptor may be required for platelet activation by low concentrations of α-thrombin.

Thrombin activates blood platelets to undergo a wide range of biochemical changes, including shape change, aggregation, thromboxane production, and secretion of granule contents [15]. Among the intracellular events activated by thrombin are the stimulation of phospholipase C, phospholipase A_2, and Na^+/H^+ exchange; it also inhibits platelet adenylate cyclase.

† Corresponding author: Steven Seiler, Ph.D., Department of Cardiovascular Biochemistry, K3119A, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543. Tel. (609) 252-4225; FAX (609) 252-6964.

Thrombin has low-, moderate-, and high-affinity binding sites on the surface of platelets [16]. The differential responses of platelets to low and high concentrations of thrombin have caused some to suggest that thrombin activation of platelets occurs through several pathways [17–19]. Also, rat and dog platelets are not activated by SFLLR-peptides, which raises the question of which of the platelet responses are linked to the cloned thrombin tethered-ligand receptor.

Scarborough and coworkers have suggested that Mpa-peptide* is a potential thrombin receptor antagonist†. We have used this compound as a tool to study the actions of the thrombin receptor in platelet function [20]. The results reported here indicate that thrombin stimulation of PLA_2 activity and Na^+/H^+ exchange are also mediated through the tethered-ligand receptor. These data also indicate that the tethered-ligand receptor mediates most actions elicited by low concentrations of α-thrombin involved in human platelet activation and that these actions can be prevented by a receptor antagonist.

MATERIALS AND METHODS

Human α-, γ- and ζ-thrombins were prepared as described [21, 22]. The clotting activity and purity (as determined by SDS gel electrophoresis) of the preparations used were 3354 U/mg for α-thrombin (98% α), 55 U/mg for γ-thrombin (95% γ), and 2640 U/mg for ζ-thrombin (96% ζ). Thrombin receptor activating peptides and the thrombin exosite inhibitor peptide (BMS 180742) were synthesized by automated solid phase synthesis on a Milligen/Biosearch 9600 synthesizer using standard *t*-Boc protocols [14] purchased from ImmunoDynamics (La Jolla, CA) or Bachem (Philadelphia, PA). Mpa peptide was synthesized using a Milligen/Biosearch 9600 synthesizer with standard *t*-Boc protocols, except that Boc-amino acids were activated as 1-hydroxybenzotriazole esters, and *N*-methyl-pyrroidone was used as the solvent in the coupling steps. All peptides were synthesized as the C-terminal amides unless otherwise stated. Radiolabeled [^{14}C]arachidonic acid (50 mCi/mmol) was obtained from Amersham, (Arlington Heights, IL), and [γ-^{32}P]GTP was obtained from DuPont/NEN (Boston, MA). BCECF-AM and fura 2-AM were from Molecular Probes (Eugene, OR).

Platelet aggregation. Human platelets were prepared from blood obtained from human volunteers. Blood was withdrawn by venipuncture into 3.8% trisodium citrate, and platelet-rich plasma was obtained by centrifugation at 200 *g* for 10 min. Platelets were separated from plasma proteins by gel filtration over Sepharose CL-2B 300 [23]. The gel-filtered platelets were counted using a Serono-Baker Diagnostics System 9000 cell counter (Allentown, PA) and diluted to a concentration of 2×10^8 platelets/mL. (This results in an initial optical density of approximately 0.3 O.D. units in the microplate reader.)

Platelet aggregation was followed in a conventional aggregometer (model PAP-4C, Bio/Data Corp., Hatboro, PA) or in a microtiter plate-based assay using vortex mixing at room temperature (a modification of the previously reported procedure of Fratantoni and Poindexter [24]). The test compounds were diluted into 50 μL of sterile saline in each well of a 96-well Nunc microtiter plate. Platelet aggregation was initiated when 150 μL of GFP was added to each well. The microtiter plate was vortex mixed for 10 min at room temperature on a Sarstedt TPM-2 platform vortex mixer at a mixer setting of 700. The optical density at 405 nm was then determined (after 10 min) using a Molecular Devices UV_{max} microplate reader.

Intracellular Ca^{2+} measurements. Platelet-rich plasma was incubated with 5 mM buffered EDTA, pH 7.4, and 3 mM fura-2 AM for 45 min at 37° with gentle shaking. The platelets were then recovered by centrifugation at 900 *g* for 10 min at 24° and resuspended in 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES/NaQH (pH 7.4). The platelets were incubated with test compounds in the presence of 1 mM $CaCl_2$, at a platelet concentration of 1×10^8 cells/mL. Intracellular Ca^{2+} measurements were made using a SPEX fluorometer with a thermoregulated cuvette at 37°. The excitation wavelengths used were 340 and 380 nm, and the emission wavelength was 505 nm. Intracellular Ca^{2+} concentration was determined by calibration of the fura-2 signal. Platelets were lysed with 50 mM digitonin to determine the maximum fluorescence; 5 mM basic Na_4EGTA was added to determine the minimum fluorescence. The intracellular Ca^{2+} concentrations were determined using software provided by SPEX, with a dissociation constant of 224 nM for fura-2 and Ca^{2+} [25].

GTPase measurements. Human platelet concentrates obtained from the Interstate Blood Bank, Inc. (Philadelphia, PA) were pooled and centrifuged at 132 *g* for 10 min at 4° to remove remaining red cells. The platelet-rich plasma was collected and supplemented with 5 mM EDTA. The platelets were recovered by centrifugation at 2200 *g* for 20 min at room temperature. The platelet pellets were resuspended in wash buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl, pH 7.5) and gently washed three times. Then the platelets were homogenized manually using 20 strokes of a glass/glass homogenizer in ice-cold 5 mM EDTA, 5 mM Tris–HCl, pH 7.5. The homogenate was centrifuged at 31,000 *g* for 20 min at 4°. The membrane pellet was resuspended in 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol and distributed in 1-mL aliquots of 2 mg protein/mL and stored at −70°.

* Abbreviations: Mpa-peptide, 3-mercapto-propionyl-Phe - Cha - Cha - Arg - Lys - Pro - Asn - Asp - Lys - amide); BMS180742, succinyl-Phe-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-cyclohexylalanine - Gln; I-BOP, 7 - [3 - [3 - hydroxy - 4-(4-iodo-phen-oxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-[1*S*-[1α,2α(Z),3β(1E,3S*),4α]]-5-heptenoic acid; BCECFAM, 2′,7′-bis(carboxyethyl)5,6,-carboxyfluorescein aceto-oxymethyl ester; GFP, gel-filtered platelets; and PRP, platelet-rich plasma.

† Scarborough RM, Teng W, Naughton MA, Rose JW, Alves V, Arfsten A, Ramakrishnan V and Blackhart B, Thrombin receptor antagonists derived from "tethered ligand" agonist peptides. Abstract No. P632, 13th American Peptide Symposium, Edmonton, Canada, 1993.

Protein content was determined using the method of Bradford [26]. On the day of the assay, the platelet membranes were diluted with ice-cold 10 mM triethanolamine HCl, 5 mM EDTA, pH 7.4, and collected by centrifugation for 10 min at 39,000 *g* at 4°. The supernatant was removed, and the pellet resuspended in the same medium. The membranes were washed two times in this manner and then resuspended in ice-cold 10 mM triethanolamine HCl plus 100 mM NaCl, pH 7.4.

The GTPase reaction was started by the addition of the membranes (30 μg in a volume of 50 μL) to 50 μL of reaction medium [27]. The final reaction contained 0.4 μM GTP ([γ-^{32}P]GTP, 5×10^5dpm/mL), 100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ATP, 5 mM phosphocreatine (Tris salt), 100 U/mL creatine phosphokinase, 0.2% bovine serum albumin, 50 mM imidazole, pH 7.3, and the indicated compounds. The reaction was carried out for 10 min at room temperature (22–24°) and stopped with 750 μL of cold 5% (w/v) activated charcoal (powder) in 20 mM phosphoric acid. The tubes were centrifuged for 20 min at maximum speed at 4° in an Eppendorf 5415C Microfuge, and 450 μL of the supernatant was added to 5 mL scintillation fluid (Eco-Lite) and counted in a scintillation counter.

Intracellular pH measurement. PRP was prepared from citrated whole blood, and EGTA was added so that the PRP also contained 1 mM EGTA (in early experiments the PRP and probe incubation media also contained 1 μM prostaglandin E_1; however, it was later removed without effect). The platelets were recovered by centrifugation at 750 *g* for 15 min (22°), and the platelets were resuspended in 138 mM NaCl, 0.3 mM Na_2HPO_4, 4 mM $NaHCO_3$, 5 mM KCl, 0.3 mM KH_2PO_4, 5.6 mM D-glucose, 2 mM phosphocreatine, 50 U/mL phosphocreatine kinase, 100 nM SQ 29548, 1 μM ketanserin, 0.6 mM EGTA, 1 mM $MgCl_2$, 0.2% BSA, 25 mM HEPES (pH 7.4), and 2 μM BCECF-AM and incubated in a shaking water bath at 37° for 30 min. The platelets were diluted 15-fold and incubated for an additional 10 min at 37°. Then they were sedimented at 350 *g* for 10 min, and gently resuspended in the same buffer without BCECF-AM. Platelets were counted using a Baker Counter and diluted to 2×10^8 platelets/mL where they were maintained at room temperature. The platelets were incubated in a 1.6-mL volume cuvette in a final platelet concentration of 1×10^8 platelets/mL. Intracellular pH measurements were made using a SPEX fluorometer with a thermoregulated cuvette at 37°. The excitation wavelengths used were 438 and 500 nm, and the emission wavelength was 530 nm. Intracellular pH was determined by calibration of the BCECF signal. Platelets were lysed with 50 μM digitonin and the pH/fluorescence curve for each sample was taken to enable calibration of fluorescence intensity to pH.

PLA_2 assays. Blood was collected in acid citrate, and PRP was obtained by centrifugation for 10 min at 200 *g*. To the PRP was added 1 mM EGTA, 100 nM SQ 29548, 1 μM prostaglandin E_1, 1 μM ketanserin, 100 μM indomethicin, 2 mM phosphocreatine, and 50 U/mL of creatine kinase. The PRP was centrifuged (750 *g* for 10 min), and the platelets were resuspended in a buffer containing 1 mM EGTA, 100 nM SQ 29548, 1 μM ketanserin, 2 mM phosphocreatine, 50 U/mL creatine kinase, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 3 mM HEPES, pH 7.4, 3.5 mg/mL BSA, and 1.4 μCi [^{14}C]arachidonic acid/mL of platelets. The platelets were incubated for 1 hr at 37° and then diluted and washed in the same buffer containing the above inhibitors without the radiolabeled arachidonic acid. The platelets were washed by centrifugation (750 *g* for 10 min), resuspended, and then sedimented. The washed platelets were divided into 500-μL aliquots and were incubated at 37° in an aggregometer with stirring (700 rpm) for 6 min with the appropriate test compounds. The reactions were terminated with the addition of 1.88 mL of ice-cold chloroform : methanol (2 : 1) followed by the addition of 0.6 mL of chloroform and 0.6 mL of 0.1% formic acid. The layers were separated by centrifugation. The non-aqueous phase was evaporated (using a SpeedVac), and the remaining material was dissolved in 200 μL of methanol : chloroform. The samples were spotted on thin-layer chromatography plates, that were developed in $CHCl_3$: methanol : acetic acid (90 : 5 : 5). The spots containing the radiolabeled arachidonic acid were identified using a scanning radioactivity detector. The spots containing the free radiolabeled arachidonic acid were scraped and counted in a liquid scintillation counter.

RESULTS

Mpa-peptide actions on platelets. A thrombin receptor antagonist, such as was described recently [28], should be useful in probing the signaling pathways involved in thrombin-induced platelet activation. We sought to determine the utility of this antagonist in platelet function studies and also to determine what platelet responses to thrombin and SFLLR-peptides were affected by the antagonist. The thrombin receptor antagonist Mpa-peptide inhibited SFLLR-peptide-induced but not collagen-induced platelet aggregation (Fig. 1). Using platelet aggregation as the assay, the ability of the inhibitor to inhibit both SFLLR-peptide and thrombin-induced platelet aggregation depended on the agonist concentration. Mpa-peptide-inhibited SFLLRNP and thrombin-induced platelet aggregation could be overcome by higher concentrations of the platelet-activating agonists (Fig. 2). When the aggregation data were subjected to Schild plot analysis, a linear plot was obtained with similar slopes and pA_2 values for the inhibition of both thrombin-induced and peptide-induced platelet aggregation. For both thrombin and SFLLRNP as agonists, the slopes were between 1.3 and 1.9 and the pA_2 between 4.5 and 5.0.

The Mpa-peptide had little effect on collagen-induced platelet aggregation, suggesting some specificity for the thrombin receptor. The Mpa-peptide also failed to inhibit thrombin proteolytic activity (measured using 10 μM D-Phe-Pip-Arg-pNA as the substrate, as described in Ref. 29). The IC_{50} for thrombin inhibition was observed to be much greater than the highest concentration tested (300 μM).

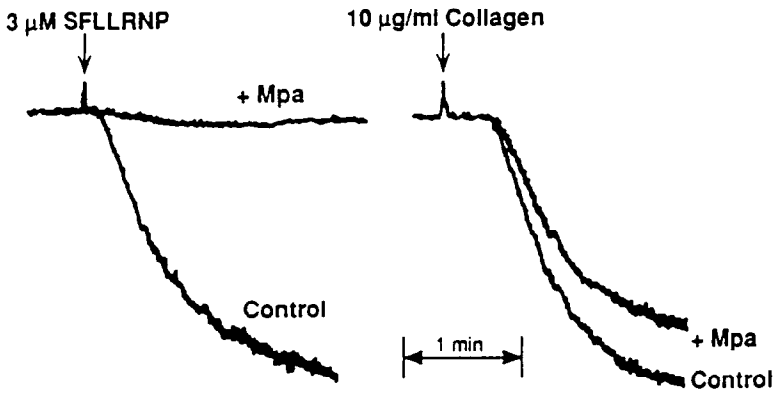

Fig. 1. Inhibition of SFLLRNP-induced but not collagen-induced platelet aggregation by Mpa-peptide. Human gel-filtered platelets were subject to aggregation measurements using a conventional four-channel aggregometer. Platelets were stirred (1100 rpm) at 37° and preincubated with or without 200 μM Mpa peptide. At the arrow 3 μM SFLLRNP or 10 μg/mL collagen was added. These data are representative of 3–4 experiments giving similar results.

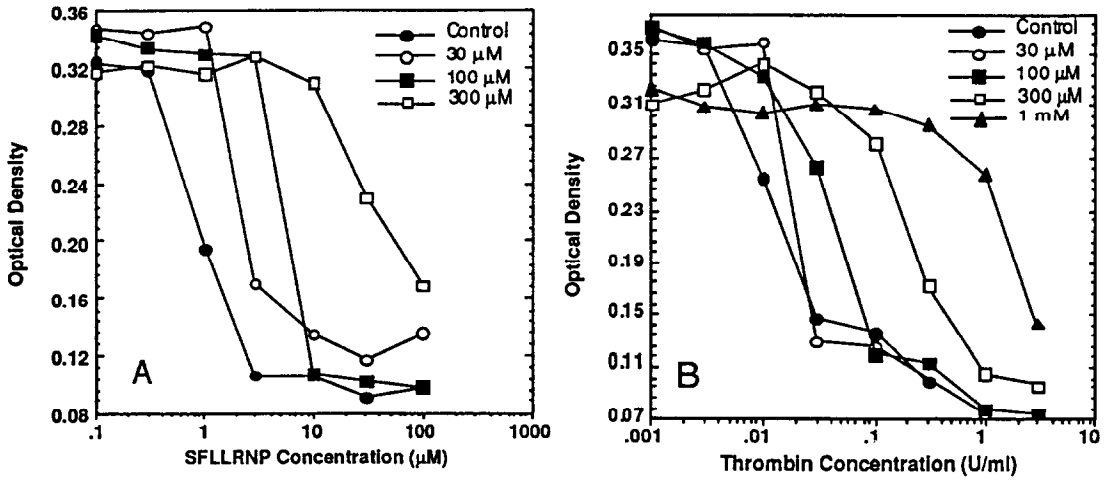

Fig. 2. Inhibition of SFLLRNP-induced (A) and α-thrombin-induced (B) platelet aggregation by the receptor antagonist Mpa-peptide. Aliquots of gel-filtered platelets were incubated with the various concentrations of Mpa for 10 min (at room temperature). Then 150 μL of these treated gel-filtered platelets was added to the indicated concentrations of SFLLRNP (A) or α-thrombin (B) in 50 μL of 0.9% saline, and the 96-well plates were vortex mixed for 10 min. These data are representative of 3 experiments giving similar results.

Thrombin and SFLLR-peptides are known to cause increases in intracellular Ca^{2+} [1]. Mpa-peptide also prevented thrombin and SFLLRNP-induced calcium mobilization in a concentration-dependent fashion with little effect on I-BOP-induced Ca^{2+} mobilization (Fig. 3). This indicated that Mpa-peptide inhibited platelet aggregation at a step prior to calcium mobilization.

The SFLLR-peptide stimulated GTPase in isolated platelet membranes (Fig. 4). The peptide-stimulated GTPase activity could be inhibited by the antagonist Mpa-peptide as indicated by the ability of Mpa-peptide to increase the amount of SFLLR-peptide required to stimulate GTPase activity (Fig. 4). A 10 μM concentration of I-BOP caused a 40% increase in GTPase activity, which was not affected by 100 μM Mpa-peptide (not shown). Taken together, these studies indicate that Mpa-peptide inhibits thrombin receptor activation, which accounts for the ability of Mpa-peptide to inhibit SFLLRNP-induced platelet aggregation.

Na^+/H^+ *exchange activation.* When platelets are treated with thrombin or SFLLR-peptides, there is a rapid initial apparent acidification followed by a subsequent prolonged alkalization. Virtually all of the cellular alkalization (both thrombin-stimulated and peptide-stimulated) could be inhibited completely by 100 μM *N,N*-hexamethylene amiloride,

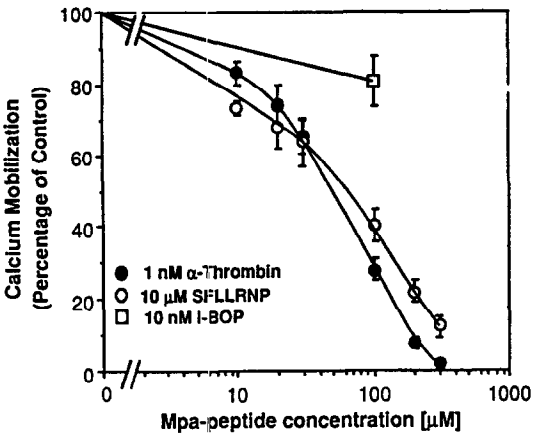

Fig. 3. Inhibition of 1 nM α-thrombin- and 10 μM SFLLRNP- induced Ca^{2+} mobilization by Mpa-peptide. Intracellular calcium was measured using fluorescence of intracellular fura 2. The control changes in intracellular Ca^{2+} were 269 ± 87.6 nM for the thrombin, 225 ± 53.2 nM for SFLLRNP and 219 ± 27.8 nM for that mobilized by 10 nM I-BOP. The effect on 10 nM I-BOP-induced platelet Ca^{2+} mobilization is also shown. Each value is the mean ± SEM (N = 3).

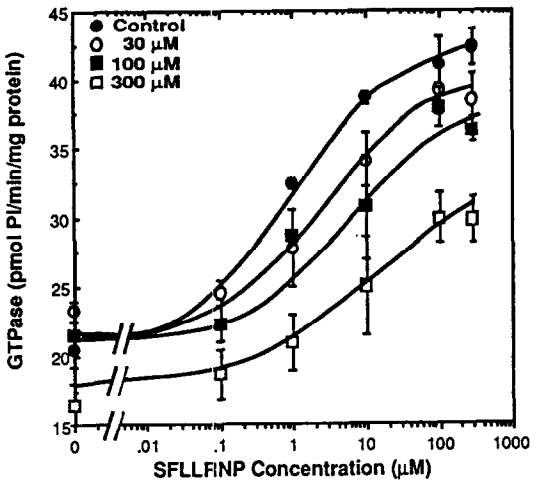

Fig. 4. Stimulation of GTPase activity by SFLLRNP-peptide and inhibition by the indicated concentrations of Mpa-peptide. Each value is the mean ± SEM (N = 3).

indicating that the alkalization is via activation of the Na^+/H^+ exchange mechanism. Both α-thrombin and SFLLR-peptide stimulated intracellular pH elevation to approximately the same degree (Fig. 5). Activation of Na^+/H^+ exchange by low concentrations of thrombin required the presence of a functioning thrombin anion-binding exosite, since low concentrations of α-thrombin and ζ-thrombin, but not γ-thrombin, could stimulate Na^+/H^+ exchange (Fig. 6). The activation of Na^+/H^+ exchange by α- and ζ-thrombin could be prevented by the anion-binding exosite inhibitor BMS 180742 (Fig. 6). The stimulation of the cytoplasmic alkalization was specific for peptides that mimic the new N-terminus after thrombin activation. SFLLR-containing peptides stimulated activation, whereas peptides with the first two amino acids reversed or acetylated-SFLLRNP did not (Fig. 7). Thrombin- and SFLLR-containing peptide-stimulated Na^+/H^+ exchange activity was also inhibited by the Mpa-peptide in a concentration-dependent fashion (Fig. 8).

PLA_2 activation. Platelet PLA_2 could be stimulated by the thrombin receptor activating peptides in a concentration-dependent fashion. Figure 9 compares the abilities of thrombin and SFLLRNP to stimulate the release of radiolabeled arachidonic acid. A similar peptide with the first two amino acids reversed and the acetylated-SFLLRNP showed no stimulatory activity (data not shown).

Thrombin activation of PLA_2 required the presence of a functioning thrombin anion-binding exosite. α-Thrombin and ζ-thrombin, but not γ-thrombin, stimulated PLA_2 as measured by the release of free radiolabeled arachidonic acid (Fig. 10). The activation of PLA_2 by α- and ζ-thrombin could be prevented by the anion-binding exosite inhibitor BMS 180742 (Fig. 10). Furthermore, the thrombin receptor antagonist Mpa-peptide inhibited both the thrombin-activated and peptide-activated PLA_2 activity in washed human platelets (Fig. 11) (as indicated by the receptor antagonist increasing the concentration of α-thrombin and SFLLR-peptide required to activate the PLA_2 activity).

DISCUSSION

These studies demonstrate that activation of the tethered-ligand thrombin receptor is coupled to the activation of human platelet phospholipase A_2 activity and Na^+/H^+ exchange activation in that: (1) activation of both processes could be mimicked (to a similar degree as α-thrombin activation) by SFLLR-containing peptides; (2) the peptide activation was specific for the SFLLR activating sequence; (3) activation of the processes required thrombin's anion-binding exosite, as they were not activated by low concentrations of γ-thrombin, and the activation observed by α- and ζ-thrombin was blocked by peptides mimicking the C-terminal region of hirudin; and (4) stimulation of the processes by both thrombin and SFLLR-containing peptides was inhibited by the thrombin receptor antagonist Mpa-peptide. These studies are all consistent with the hypothesis that thrombin stimulation of PLA_2 activity and Na^+/H^+ exchange occurs via activation of the thrombin tethered-ligand receptor.

Activation of platelet Na^+/H^+ exchange, stimulation of PLA_2, and inhibition of adenylate cyclase were characterized previously as a "high-affinity" thrombin activation pathway [19]. We had shown previously that the thrombin tethered-ligand receptor in platelets is coupled to inhibition of adenylate cyclase [11]. In the present study, we demonstrated that the platelet PLA_2, Na^+/H^+ exchange and GTPase activity are also stimulated through the

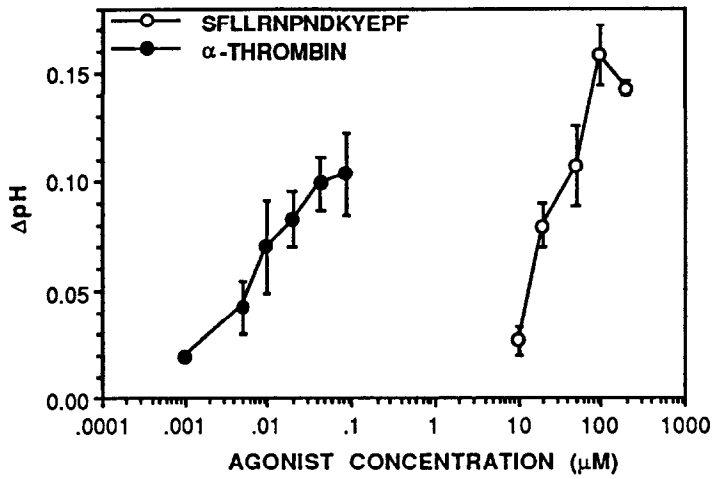

Fig. 5. Stimulation of platelet Na^+/H^+ exchange by thrombin and the receptor activating peptide SFLLRNPNDKYEPF. Washed human platelets were labeled with a membrane permeable fluorescent dye, BCECF-AM, loading the cells with BCECF. Changes in intracellular pH (pH_i) were then measured using a SPEX fluorometer, and the actual pH_i was determined following an internal calibration procedure. Each value is the mean ± SEM (N = 3).

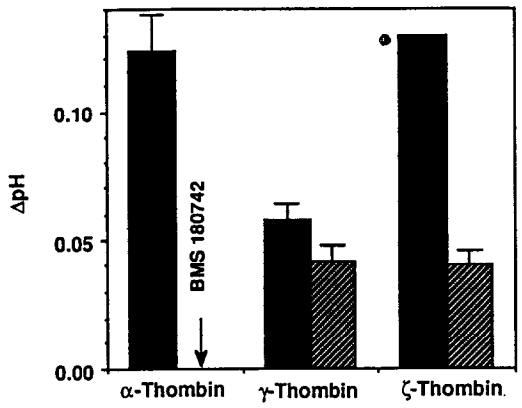

Fig. 6. Stimulation of platelet Na^+/H^+ exchange by a 20 nM concentration of each thrombin (solid bars) and inhibition by 20 μM thrombin exosite inhibitor BMS 180742 (hatched bars). Washed human platelets were prepared and then labeled with BCECF-AM as described in Materials and Methods. Changes in intracellular pH (pH_i) were then measured, and the actual pH_i was determined using a calibration procedure. Each value is the mean ± SEM (N = 3).

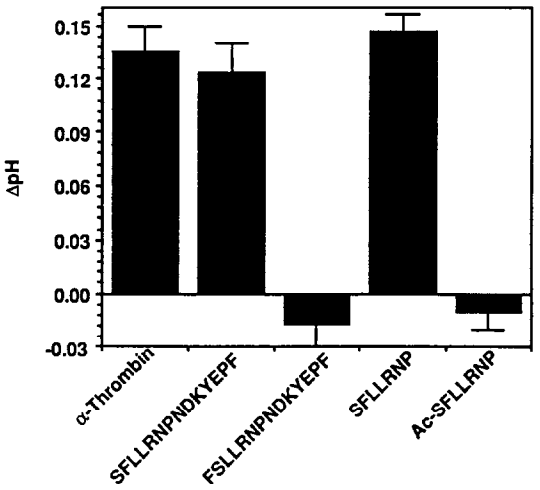

Fig. 7. Peptide specificity of platelet Na^+/H^+ exchange activation. Na^+/H^+ exchange activity was measured as described in Materials and Methods. The platelets were treated with a 100 μM concentration of the indicated peptides or 10 nM α-thrombin. Each value is the mean ± SEM (N = 3).

tethered-ligand thrombin receptor. Studies by other investigators indicate that this receptor is also involved in platelet phospholipase C activation [10], Ca^{2+} mobilization [1] and intracellular tyrosine phosphorylation [8]. Taken together, most of thrombin's platelet activating intracellular signaling pathways leading to platelet aggregation are activated via the thrombin tethered-ligand receptor in human platelets and are prevented when this receptor is blocked.

Activation of the platelet Na^+/H^+ exchange activity has been observed with elevation of the cytoplasmic Ca^{2+} levels [30–32], activation of protein kinase C [33, 34], and direct activation by G-proteins [35]. Coupling of the thrombin tethered-ligand receptor to Na^+/H^+ exchange activation is consistent with any or all of the proposed mechanisms for Na^+/H^+ exchange activation, since thrombin receptor activation causes G-protein activation (as shown in this study by stimulation of GTPase activity), PLC activation [10], as well as Ca^{2+} mobilization [1].

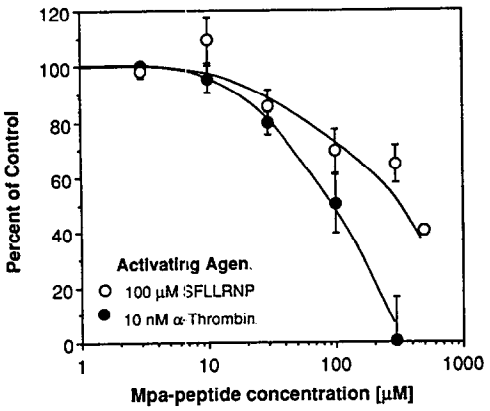

Fig. 8. Inhibition of α-thrombin- and SFLLRNP-activated platelet cytoplasmic alkalization by Mpa-peptide. Intracellular pH of washed human platelets was measured using the fluorescent dye BCECF, and the average resting pH was 7.12 ± 0.03 (N = 6). The control changes in intracellular pH were 0.13 ± 0.01 for 10 nM α-thrombin and 0.10 ± 0.02 for 100 μM SFLLRNP. Each value is the mean ± SEM (N = 3–6).

Previous studies had indicated that most of the arachidonic acid mobilization resulting from thrombin activation of platelets results from PLA_2 activation [33, 36–38]. There is considerable evidence that the PLA_2 activation is controlled, at least in part, by a G-protein coupled process [36, 38, 39]. Platelet arachidonate mobilization may also be stimulated by elevated intracellular Ca^{2+} [38, 40, 41] and activation of protein kinase C [42]. PLA_2 activation is consistent with thrombin tethered-ligand thrombin receptor activation, which is known to stimulate GTPase, PLC activation, and Ca^{2+} mobilization.

Mpa-peptide was used as a thrombin receptor antagonist in these studies. This compound was capable of preventing both thrombin- and SFLLR-peptide-induced platelet aggregation and calcium mobilization, as well as platelet membrane GTPase activity. This inhibitor was also capable of inhibiting thrombin- and SFLLR-peptide-induced PLA_2 and Na^+/H^+ exchange activation.

It is not likely that the inhibition of thrombin and SFLLR-peptide activation of platelets is due to desensitization of thrombin receptor response by a partial agonist. Activation of platelet membrane GTPase has proven to be very sensitive to the identification of partial agonists and not directly subject to desensitization by agonists. The peptide YFLLRNP was reported to be a thrombin receptor antagonist [43]. However, we found that this peptide could stimulate both GTPase (to about 50% of the maximal activity of SFLLRNP) and platelet aggregation, using the microplate method described here*, which indicates that YFLLRNP is a partial agonist. In this regard, the Mpa-peptide appears to be a more complete antagonist than YFLLRNP in that the Mpa-peptide showed no ability to stimulate platelet membrane GTPase assay, but inhibited GTPase stimulated by thrombin receptor activation. Thus, the inhibition of platelet responses is not likely to be due to desensitization but instead is most easily explained by direct receptor antagonism.

* Seiler SM and Peluso M, unpublished data.

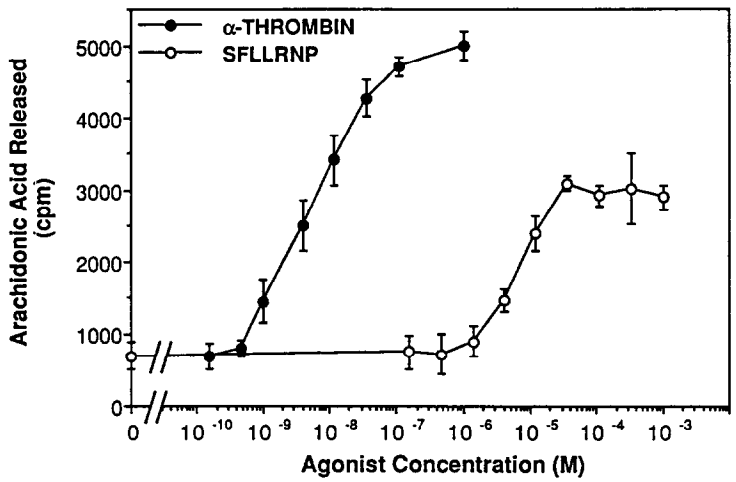

Fig. 9. Thrombin and SFLLR-peptide stimulation of platelet PLA_2 activity. Washed human platelets were incubated with radiolabeled arachidonic acid and then washed again to remove excess radiolabel. The radiolabeled platelets were incubated in the presence of 1 mM EGTA, 100 nM SQ 29548, 1 μM ketanserin, 2 mM phosphocreatine, 50 U/mL creatine kinase, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 3 mM HEPES, pH 7.4, and 3.5 mg/mL BSA and then treated with the indicated concentrations of thrombin or peptide for 6 min at 37°. The reaction was stopped by extracting the lipids into organic solvent. The lipids were fractionated by TLC and quantitated by scraping and liquid scintillation counting of free arachidonic acid. Each value is the mean ± SD of 5 determinations.

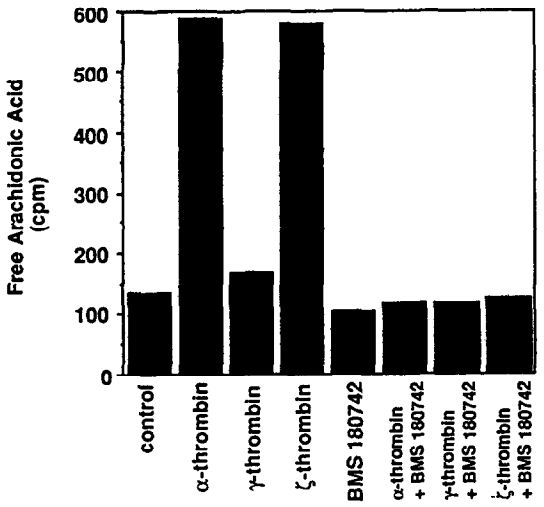

Fig. 10. Effect of 5 nM α-, ζ- and γ-thrombin on platelet PLA_2 activity with and without thrombin exosite inhibitor (20 μM BMS 180742). Human platelets were incubated with radiolabeled arachidonic acid and washed again to remove excess radiolabel. The platelets were incubated in the presence of 1 mM EGTA, 100 nM SQ 29548, 1 μM ketanserin, 2 mM phosphocreatine, 50 U/mL creatine kinase, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 3 mM HEPES, pH 7.4, and 3.5 mg/mL BSA and treated with thrombins ± 20 μM BMS 180742 for 6 min at 37°. The reaction was stopped by extracting the lipids into organic solvent. The lipids were fractionated by TLC. Free arachidonic acid was quantitated by scraping and liquid scintillation counting. This graph is representative of 3 experiments with similar results.

The inhibition of platelet response by Mpa-peptide is surmountable, as indicated by the ability of the Mpa-peptide to shift the SFLLR-peptide and thrombin concentration–response relationships (observed with Mpa-peptide antagonism of platelet aggregation, GTPase and PLA_2 activation), and the inhibitory effects of the Mpa-peptide can be overcome with higher concentrations of thrombin or SFLLR-peptide. The surmountable nature would suggest competitive antagonism if these experiments represented equilibrium conditions of ligand–receptor interactions and slopes of Schild plots were equal to unity. However, Schild analysis of platelet aggregation and GTPase inhibition data gives slopes of 1.3 to 1.9, suggesting that the antagonism is not a simple competitive interaction, which is difficult to characterize. Taken together, these studies suggest that the compound (Mpa-peptide) is not likely to act via desensitization, but instead is a direct-acting, surmountable thrombin receptor antagonist.

Relatively high concentrations of Mpa-peptide are required to be effective. Therefore, it is unlikely to be specific for the thrombin receptor, even though the peptide had little effect on collagen or I-BOP-induced platelet aggregation, and had little effect on I-BOP-induced platelet Ca^{2+} mobilization and I-BOP-stimulated GTPase. In human umbilical vein endothelial cells, Mpa-peptide inhibited both thrombin and SFLLR-peptide stimulation of prostacyclin production, expected of a thrombin receptor antagonist (unpublished data). However, it also causes small, but measurable, Ca^{2+} mobilization in these cells, suggesting that Mpa-peptide may have some partial agonist character in that cell type. Mpa-peptide also caused a small increase in intracellular Ca^{2+} in CHRF 288 cells and cultured rat aortic smooth muscle cells, again suggesting that this compound acted as a partial agonist in this cell type (data not shown). However, the Mpa-peptide caused no Ca^{2+} mobilization and appears more as a complete antagonist in human platelets. The reasons for the cell-specific differences in response are not clear at present and are currently under investigation.

Comparisons of effects of equimolar α- and γ-thrombin and the thrombin anion-binding exosite inhibitors were used in these studies to implicate the thrombin anion-binding exosite shown to be utilized in tethered-ligand receptor activation by low

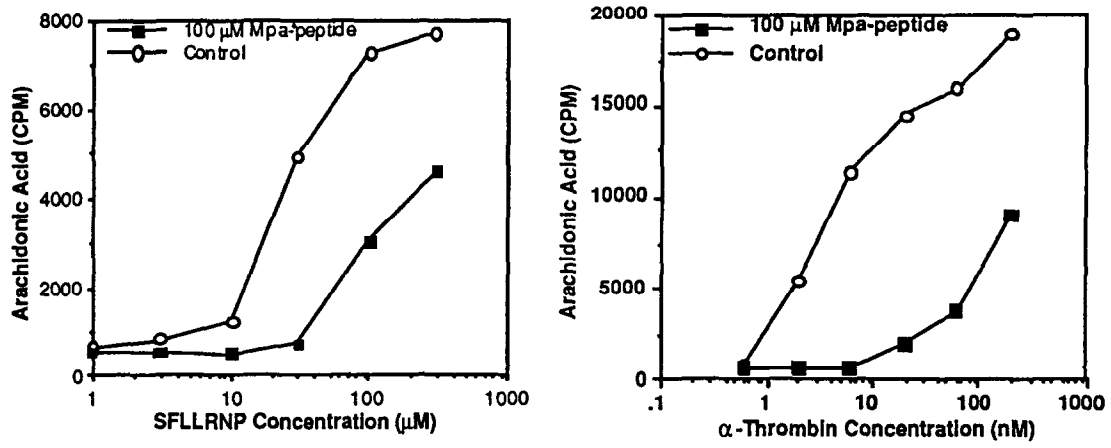

Fig. 11. Inhibition of platelet PLA_2 activity stimulated by the peptide SFLLRNP (left panel) or α-thrombin (right panel). Platelets prelabeled with [^{14}C]arachidonic acid were incubated with increasing concentrations of the activating agonist in the presence (■) or absence (○) of 100 μM Mpa-peptide. Each graph is representative of 3 or 4 determinations.

concentrations of α-thrombin. However, higher concentrations of γ-thrombin (>50-fold higher concentrations than required for α-thrombin to activate the same processes) are capable of activating the thrombin tethered-ligand receptor [13]. It is also likely that those much higher concentrations of γ-thrombin could also stimulate PLA_2 and Na^+/H^+ exchange activity as well as inhibiting adenylate cyclase activity.

In summary, many of the intracellular signals activated by low concentrations of thrombin appear mediated by the thrombin tethered-ligand receptor in human platelets. These include activation of G-proteins, activation of phospholipase C [10], Ca^{2+} mobilization [1], activation of Na^+/H^+ exchange and stimulation of PLA_2, inhibition of adenylate cyclase [11], and tyrosine phosphorylation [8]. This does not rule out the possibility that other thrombin receptors and activation pathways exist nor does it imply that platelets from other species respond the same as human platelets, but it does emphasize the importance of this receptor in human platelet activation.

Acknowledgements—We wish to thank Ving G. Lee and Jack Hunt for peptides used in the early portion of this study. We would also like to thank Daniel Roberts and Karen Hartl for technical assistance and Dr. George Zavoico for help with the Na^+/H^+ exchange studies. This work was supported, in part, by NIH Grant HL13160 (J.W.F.).

REFERENCES

1. Vu T-KH, Hung DT, Wheaton VI and Coughlin SR, Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**: 1057–1068, 1991.
2. Rasmussen UB, Vouret-Craviari V, Jallat S, Schlesinger Y, Pages G, Pavirani A, Lecocq J-P, Pouyssegur J and Van Obberghen-Schilling E, cDNA cloning and expression of a hamster α-thrombin receptor coupled to Ca^{2+} mobilization. *FEBS Lett* **288**: 123–128, 1991.
3. Zhong C, Hayzer DJ, Corson MA and Runge MS, Molecular cloning of the rat vascular smooth muscle thrombin receptor: Evidence for *in vitro* regulation by basic fibroblast growth factor. *J Biol Chem* **267**: 16975–16979, 1992.
4. Vu T-KH, Wheaton VI, Hung DT and Coughlin SR, Domains specifying thrombin–receptor interaction. *Nature* **351**: 674–677, 1991.
5. Liu L-W, Vu T-KH, Esmon CT and Coughlin SR, The region of the thrombin receptor resembling hirudin binds to thrombin and alters enzyme specificity. *J Biol Chem* **266**: 16977–16980, 1991.
6. Chao BH, Kalkunte S, Maraganore JM and Stone SR, Essential groups in synthetic agonist peptides for activation of the platelet thrombin receptor. *Biochemistry* **31**: 790–796, 1992.
7. Scarborough RM, Naughton MA, Teng W, Hung DT, Rose J, Vu T-KH, Wheaton VI, Turck CW and Coughlin SR, Tethered ligand agonist peptides: Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J Biol Chem* **267**: 13146–13149, 1992.
8. Vassallo RR Jr, Kieber-Emmons T, Cichowski K and Brass LF, Structure–function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J Biol Chem* **267**: 6081–6085, 1992.
9. Vouret-Craviari V, Van Obberghen-Schilling E, Rasmussen UB, Pavirani A and Pouyssegur J, Synthetic α-thrombin receptor peptides activate G protein-coupled signaling pathways but are unable to induce mitogenesis. *Mol Biol Cell* **3**: 95–102, 1992.
10. Huang R-S, Sorisky A, Church WR, Simons ER and Rittenhouse SE, "Thrombin" receptor-directed ligand accounts for activation by thrombin of platelet phospholipase C and accumulation of 3-phosphorylated phosphoinositides. *J Biol Chem* **266**: 18435–18438, 1991.
11. Seiler SM, Michel IM and Fenton JW II, Involvement of the "tethered-ligand" receptor in thrombin inhibition of platelet adenylate cyclase. *Biochem Biophys Res Commun* **182**: 1296–1302, 1992.
12. Brass LF, Vassallo RR Jr, Belmonte E, Ahuja M, Cichowski K and Hoxie JA, Structure and function of the human platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J Biol Chem* **267**: 13795–13798, 1992.
13. Hung DT, Vu T-KH, Wheaton VI, Ishii K and Coughlin SR, Cloned platelet thrombin receptor is necessary for thrombin-induced platelet activation. *J Clin Invest* **89**: 1350–1353, 1992.
14. Seiler SM, Goldenberg HJ, Michel IM, Hunt JT and Zavoico GB, Multiple pathways of thrombin-induced platelet activation differentiated by peptide desensitization and by thrombin exosite inhibitors. *Biochem Biophys Res Commun* **181**: 636–643, 1991.
15. Siess W, Molecular mechanisms of platelet activation. *Physiol Rev* **69**: 58–178, 1989.
16. Harmon JT and Jamieson GA, Platelet activation by thrombin in the absence of the high-affinity thrombin receptor. *Biochemistry* **27**: 2151–2157, 1988.
17. McGowan EB and Detwiler TC, Modified platelet responses to thrombin. Evidence for two types of receptors or coupling mechanisms. *J Biol Chem* **261**: 739–746, 1986.
18. Yamamoto N, Greco NJ, Barnard MR, Tanoue K, Yamazaki H, Jamieson GA and Michelson AD, Glycoprotein Ib (GPIb)-dependent and GPIb-independent pathways of thrombin-induced platelet activation. *Blood* **77**: 1740–1748, 1991.
19. Greco N and Jamieson GA, High and moderate affinity pathways for α-thrombin-induced platelet activation. *Proc Soc Exp Biol Med* **198**: 792–799, 1991.
20. Seiler SM, Michel IM, Goldenberg H, Gamberdella M, Roberts DGM, Riexinger D, Natarajan S, Fenton JW II and Zavoico GB, Stimulation of the platelet thrombin receptor causes activation of phospholipase A_2 and Na^+/H^+ exchange. *Thromb Haemost* **69**: 1148, 1993.
21. Fenton JW II, Fasco MJ, Stackrow AB, Aronson DL, Young AM and Finlayson JS, Human thrombins: Production, evaluation, and properties of α-thrombin. *J Biol Chem* **252**: 3587–3598, 1977.
22. Brezniak DV, Brower MS, Witting JI, Walz DA and Fenton JW II, Human α- to ζ-thrombin cleavage occurs with neutrophil cathepsin G or chymotrypsin while fibrinogen clotting activity is retained. *Biochemistry* **29**: 3536–3542, 1990.
23. Timmons S and Hawiger J, Isolation of human platelets by albumin gradient and gel filtration. *Methods Enzymol* **169**: 11–21, 1989.
24. Fratantoni JC and Poindexter BJ, Measuring platelet aggregation with microplate reader. A new technical approach to platelet aggregation studies. *Am J Clin Pathol* **94**: 613–617, 1990.
25. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440–3450, 1985.
26. Bradford MM, A rapid and sensitive method for the

quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
27. Shenker A, Goldsmith P, Unson CG and Spiegel AM, The G-protein coupled to the thromboxane A_2 receptor in human platelets is a member of the novel G_q family. *J Biol Chem* **266**: 9309–9313, 1991.
28. Scarborough R, Naughton MA, Teng W, Hung DT, Rose JW, Alves V and Arfsten A, A thrombin receptor antagonist derived from tethered ligand agonist peptides. *Circulation* **86**: 1–151, 1992.
29. Balasubramanian N, St. Laurent DR, Federici ME, Meanwell NA, Wright JJ, Schumacher WA and Seiler SM, Active site-directed synthetic thrombin inhibitors: Synthesis, *in vitro* and *in vivo* activity profile of BMY 44621 and analogs. An examination of the role of the amino group in the D-Phe-Pro-Arg-H series. *J Med Chem* **36**: 300–303, 1993.
30. Siffert W and Akkerman JWN, Na^+/H^+ exchange as a modulator of platelet activation. *Trends Biochem Sci* **13**: 148–151, 1988.
31. Zavoico GB, Cragoe EJ Jr and Feinstein MB, Regulation of intracellular pH in human platelets. *J Biol Chem* **261**: 13160–13167, 1986.
32. Kimura M, Lasker N and Aviv A, Cyclic nucleotides attenuate thrombin-evoked alterations in parameters of platelet Na/H antiport: The role of cytosolic Ca. *J Clin Invest* **89**: 1121–1127, 1992.
33. Livne AA, Aharonovitz O, Fridman H, Tsukitani Y and Markus S, Modulation of Na^+/H^+ exchange and intracellular pH by protein kinase C and protein phosphatase in blood platelets. *Biochim Biophys Acta* **1068**: 161–166, 1991.
34. Livne AA, Sardet C and Pouyssegur J, The Na^+/H^+ exchanger is phosphorylated in human platelets in response to activating agents. *FEBS Lett* **284**: 219–222, 1991.
35. Siffert W, Jacobs KH and Akkerman JWN, Sodium fluoride prevents receptor- and protein kinase C-mediated activation of the human platelet Na^+/H^+ exchanger without inhibiting its basic pH_i-regulating activity. *J Biol Chem* **265**: 15441–15448, 1990.
36. Nakashima S, Tohmatsu T, Hattori H, Suganuma A and Nozawa Y, Guanine nucleotides stimulate arachidonic acid release by phospholipase A_2 in saponin-permeabilized human platelets. *J Biochem (Tokyo)* **101**: 1055–1058, 1987.
37. Fuse I and Tai H-H, Stimulations of arachidonate release and inositol-1,4,5-trisphosphate formation are mediated by distinct G-proteins in human platelets. *Biochem Biophys Res Commun* **146**: 659–665, 1987.
38. Kajiyama Y, Murayama T and Nomura Y, Pertussis toxin-sensitive GTP-binding proteins may regulate phospholipase A_2 in response to thrombin in rabbit platelets. *Arch Biochem Biophys* **274**: 200–208, 1989.
39. Kajiyama Y, Murayama T, Kitamura Y and Nomura Y, Possible involvement of different GTP-binding proteins in noradrenaline- and thrombin-stimulated release of arachidonic acid in rabbit platelets. *Biochem J* **270**: 69–75, 1990.
40. Rittenhouse SE and Horne WC, Ionomycin can elevate intraplatelet Ca^{2+} and activate phospholipase A without activating phospholipase C. *Biochem Biophys Res Commun* **123**: 393–397, 1984.
41. Authi KS, Evenden BJ and Crawford N, Metabolic and functional consequences of introducing inositol 1,4,5-trisphosphate into saponin-permeabilized human platelets. *Biochem J* **233**: 709–718, 1986.
42. Halenda SP, Banga HS, Zavoico GB, Lau L-F and Feinstein MB, Synergistic release of arachidonic acid from platelets by activators of protein kinase C and Ca^{2+} ionophores. Evidence for the role of protein phosphorylation in the activation of phospholipase A_2 and independence from the Na^+/H^+ exchanger. *Biochemistry* **28**: 7356–7363, 1989.
43. Rasmussen UB, Gachet C, Schlesinger Y, Hanau D, Ohlmann P, Van Obberghen-Schilling E, Pouyssegur J, Cazenave J-P and Pavirani A, A peptide ligand of the human thrombin receptor antagonizes thrombin and partially activates platelets. *J Biol Chem* **268**: 14322–14328, 1993.