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Early Intraplatelet Signaling Enhances the Release of Human Platelet PAR-1 and -4 Amino-Terminal Peptides in Response to Thrombin[†]

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ABSTRACT: Activation of washed human platelets initiated with α-thrombin, SFLLRN, or AYPGKF invariably results in the generation of PAR-1-(1−41) and PAR-4-(1−47). PAR-1-(1−41) and PAR-4-(1−47) are amino-terminal peptides generated when PAR-1 and -4 are cleaved in their first extracellular domains after R^{41} and R^{47}, respectively, to expose the tethered ligand domains of PAR-1 and -4. Since soybean trypsin inhibitor decreases generation of PAR-1-(1−41) and PAR-4-(1−47) and other platelet aggregation-related responses to these three agonists, but does not inactivate α-thrombin, a platelet trypsin-like proteinase apparently activates PAR-1 and -4 to propagate PAR-dependent platelet responses. This study identified the signaling pathways implicated in the generation of the platelet proteinase that in turn produces PAR-1-(1−41) and PAR-4-(1−47), to thereby drive the subsequent PAR-dependent platelet aggregation-related responses to α-thrombin, SFLLRN, or AYPGKF. Only inhibitors of signaling enzymes that prevented ATP release (forskolin, PGE_1, or BIMI-1) prevented or delayed the generation of PAR-1-(1−41) and PAR-4-(1−47) in response to all three agonists. SBTI prevented platelet aggregation initiated by α-thrombin, SFLLRN, or AYPGKF but did so less effectively when it was added 10 s after each agonist. Thus, the platelet-derived proteinase acts within 10 s of each agonist addition to generate PAR-1-(1−41) and PAR-4-(1−47). Furthermore, α-thrombin may not effectively catalyze PAR-1-(1−41) and PAR-4-(1−47) generation. We propose that unidentified ATP-dependent phosphorylation reactions catalyzed by PKC help to generate the platelet-derived proteinase that propagates human platelet PAR-1 and -4 activation by the three agonists.

Incubation of washed human platelets with SFLLRN, AYPGKF, or α-thrombin invariably results in the simultaneous generation of ELISA[1]-quantifiable PAR-1-(1−41) and PAR-4-(1−47) and platelet activation (*1, 2*). Generation of PAR-1-(1−41) and PAR-4-(1−47) and the other platelet activation-related responses to SFLLRN or AYPGKF are markedly inhibited by soybean trypsin inhibitor but not by the direct thrombin inhibitor, hirudin (*1, 2*). Soybean trypsin inhibitor (an agent unable to inactivate α-thrombin (*3*)) also prevents PAR-1-(1−41) generation and platelet activation in response to α-thrombin (*1*). BMS-200261, a specific SFLLRN antagonist (*4*), or YD3, a PAR-4 antagonist (*5*), completely inhibits all responses of washed human platelets to SFLLRN or AYPGKF, including generation of PAR-1-(1−41) and PAR-4-(1−47) (*2*). Generation of human platelet PAR-1-(1−41) and PAR-4-(1−47) in response to SFLLRN or AYPGKF (*1, 2*) suggests the involvement of one or more platelet-derived proteinases in PAR-1 and -4 activation. Consistent with this idea, aprotinin (an inhibitor of trypsin-like proteinases but not α-thrombin (*3*)) decreased platelet PAR-1 activation during coronary artery bypass grafting surgery (*6*) and platelet PAR-1 activation in response to α-thrombin *in vitro* (*7*).

The major goals of this study were to identify the intraplatelet signaling enzymes directly or indirectly involved in activation of the platelet proteinase that generates PAR-

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[1] Abbreviations: AC, adenylyl cyclase; BIMI-1, 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide; CD62, P-selectin or granule membrane protein 140; CD63, a platelet glycoprotein associated with lysosomal secretion; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; PAR-1 and PAR-4, proteinase activated receptor 1 and 4, respectively (the IUPHAR nomenclature for PAR-1 and PAR-4 are PAR_1 and PAR_4, respectively); PAR-1-(1−41), the 41-mer amino-terminal peptide generated when the first extracellular domain of PAR-1 is cleaved after R^{41} by α-thrombin to expose PAR-1 tethered ligand beginning with the sequence ^{42}SFLLRN; PAR-4-(1−47), the 47-mer amino-terminal peptide generated when the first extracellular domain of PAR-4 is cleaved after R^{47} by α-thrombin to expose PAR-4 tethered ligand beginning with the sequence ^{47}GYPGKF; PE, phycoerythrin; PGE_1, prostaglandin E_1; LY294002, 2-(4-morpholino)-8-phenyl-4*H*-1-benzopyran-4-one; PI3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; SCH 79797 dihydrochloride, a synthetic PAR-1 inhibitor; SBTI, soybean trypsin inhibitor; SD, standard deviation; SEM, standard error of the mean; tcYPGKF, *trans*-cinnamoyl-YPGKF-amide, a PAR-4 inhibitor; U73122, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione, WEDE 15, a monoclonal antibody directed against PAR-1-(51−64).

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1-(1−41) and PAR-4-(1−47) or related peptides that are also derived from the first extracellular domains of PAR-1 and -4, respectively. This was accomplished by determining how several human platelet antagonists influence PAR-1-(1−41) and PAR-4-(1−47) generation and the other aggregation-related responses (*8*) to SFLLRN, AYPGKF, or α-thrombin. The platelet antagonists evaluated are forskolin and PGE_1, both of which stimulate adenylyl cyclase activity and thereby increase intracellular [cAMP] (*9*−*11*); the PKC inhibitor bisindolylmaleimide I (BIMI-1) (*12, 13*); U73122, a phospholipase C inhibitor (*14, 15*); and LY294002 hydrochloride, a PI3-kinase inhibitor (*16, 17*). Direct evidence for PAR-1 and PAR-4 activation was obtained by quantifying the generation of PAR-1-(1−41) and PAR-4-(1−47) by specific ELISAs (*1, 2, 18*). We inferred that generation of these two peptides likely coincided with generation of PAR-1 and -4 tethered ligands *in situ* at concentrations equimolar to those of PAR-1-(1−41) and PAR-4-(1−47) produced. In addition to measuring PAR-1-(1−41) and PAR-4-(1−47) generation, the effects of these cell signaling inhibitors on CD62 and CD63 expression on the platelets, ATP and [^{14}C]serotonin secretion, changes in intracellular [Ca^{2+}], and platelet aggregation in response to SFLLRN, AYPGKF (*19*), or α-thrombin were also determined.

MATERIALS AND METHODS

Highly purified α-thrombin (lot no. 390, stock concentration of 3.07×10^{-5} M (with 97.1% activity by titration with *p*-nitrophenyl *p*-guanidinobenzoate), specific activity 2421 units/mg, 93.1% α-thrombin, 2.5% β-thrombin, 4.4% γ-thrombin) was generously provided by Dr. John Fenton II, formerly of the New York Department of Health, Albany, NY (*20*). The final dilutions of the stock used to achieve 1.0 and 0.5 nM α-thrombin were 1/30000 and 1/60000, respectively. AYPGKF was purchased from University of Calgary Peptide Services, Calgary, Alberta, Canada. YAPGKF-amide and FSLLRN-amide were purchased from Genscript, Piscataway, NJ. Soybean trypsin inhibitor (high activity), PGE_1, bisindolylmaleimide I, and U73122 were purchased from Calbiochem, La Jolla, CA. LY294002 hydrochloride was purchased from Biomol International, Plymouth Meeting, PA. WEDE 15-PE conjugated was purchased from Beckman Coulter Canada, Inc., Mississauga, Ontario, Canada. ADP, arachidonic acid, and collagen were purchased from Chronolog Corp., Havertown, PA. BMS 200261, a specific SFLLRN antagonist (*4*), was a gift from Dr. S. Seiler (Bristol-Meyer-Squibb, Princeton, NJ). YD3, a specific PAR-4 antagonist, was a generous gift from Dr. C. C. Wu (*5*). SCH 79797 dihydrochloride, a specific PAR-1 antagonist, was purchased from Tocris Bioscience, Ellisville, MO. *trans*-Cinnamoyl-YPGKF-amide was purchased from Peptides International, Louisville, KY. Other reagents, including SFLLRN-amide and forskolin, were purchased from Sigma-Aldrich, Canada Ltd., Oakville, Ontario, Canada.

Platelet Preparation. Washed platelets were prepared from fresh citrated blood from healthy volunteers and resuspended at 5×10^{11} platelets/L in a modified Tyrode's buffer (0.136 M NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, 0.1% glucose, and 0.35% bovine serum albumin, pH 7.15) at 37 °C using the procedures of Mustard et al. (*21*). The volunteer blood donors had not taken any medication known to affect platelet function in the previous 14 days and provided informed consent. Ethics approval was obtained from the Research Ethics Board, Faculty of Health Sciences, McMaster University.

Aggregation-Related Responses of Washed Platelets. A single-channel platelet aggregometer (Model 600; Chronolog Corp., Havertown, PA) was used to measure aggregation of stirred washed platelets for 3 min after each agonist was added. For serotonin-release studies, washed platelets were preloaded with [^{14}C]serotonin (1 μCi/40 mL; Amersham Biosciences Inc., Baie d'Urfé, Quebec, Canada) at 37 °C followed by treatment with 1 μM imipramine to prevent reuptake of serotonin (*21*). Expression of CD62 and CD63 on human platelets was quantified by flow cytometry using FITC-labeled CD63 antibody (Immunotech, Beckman Coulter Co., Fullerton, CA) and PE-labeled CD62 antibody (BD Biosciences, Mississauga, Ontario, Canada) as described previously (*1, 18, 22*). Agonist-induced PAR-1 cleavage at $R^{41}S^{42}$ and release of PAR-1-(1−41) into the supernatants of washed platelets were quantified by an ELISA described previously (*1, 2, 18*). Agonist-induced PAR-4 cleavage at $R^{47}G^{48}$ was also measured by an ELISA (described below) as the release of PAR-4-(1−47) into platelet supernatants.

Quantification of PAR-4-(1−47) by ELISA. Six laying hens were immunized with 20 μg of synthetic human PAR-4-(1−47) peptide (Enzyme Research Laboratories, South Bend, IN) in complete Freund's adjuvant. The chickens subsequently received booster injections of 20 μg of PAR-4-(1−47) dissolved in 1 mL of normal saline every 2 weeks. The eggs were collected daily beginning 12 weeks after the initial injection. The IgY was isolated from the egg yolks and affinity purified on a 20 mL column of PAR-4-(1−47)−Sepharose 4B (Amersham Biosciences Inc., Baie d'Urfé, Quebec, Canada) using procedures described previously (*18*). The resulting affinity-purified anti-PAR-4-(1−47)−IgY was coated onto microtiter plates at a concentration of 100 ng/mL. The PAR-4-(1−47) ELISA was similar to that described for quantifying PAR-1-(1−41) (*1, 18*). Standard curves were constructed using 4 pM to 4 nM synthetic PAR-4-(1−47) peptide, and samples were assayed in duplicate. The detector antibody, affinity-purified chicken anti-PAR-4-(1−47) IgY, was labeled with biotin using *N*-hydroxysuccinimidobiotin (Pierce, Rockford, IL) according to the manufacturer's instructions. The sensitivity limit of this ELISA was 5 pM PAR-4-(1−47). This ELISA did not detect up to 10 nM synthetic PAR1-(1−41) (Enzyme Research Laboratories, South Bend, IN). Similarly, the ELISA measuring PAR-1-(1−41) did not detect up to10 nM synthetic PAR-4-(1−47). Neither the PAR 1-(1−41) nor the PAR 4-(1−47) ELISA could detect any of the major proteins (i.e., fibrinogen, 70 μg/mL; human serum albumin, 40 μg/mL; von Willebrand factor, 2 units/mL; factor V/Va, 2 μg/mL; platelet factor 4, 5 μg/mL; fibronectin, 2 μg/mL; thrombospondin, 25 μg/mL) that are known to be released by platelets incubated with α-thrombin (*23*).

Measurement of Changes in Intracellular [Ca^{2+}]. For intracellular calcium mobilization studies, 10 μM Fura-PE3 (AM) (TEF Laboratories, Austin, TX) was added to the platelet-rich plasma and incubated for 45 min at room temperature in the dark, followed by the washing procedure outlined above for isolating washed platelets. Washed

Table 1: Generation of PAR-1-(1-41) and PAR-4-(1-47) by Washed Human Platelets in Response to Platelet Agonists[a]

agonist	[PAR-1-(1−41)] (nM)	[PAR-4-(1−47)] (nM)
(A) Peptides Generated in 10 s		
none	0.02 ± 0.01	0.02 ± 0.01
10 μM SFLLRN	0.77 ± 0.13	0.17 ± 0.06
100 μM AYPGKF	0.91 ± 0.13	0.20 ± 0.08
500 μM arachidonate	0.71 ± 0.13	0.23 ± 0.12
(B) Peptides Generated in 180 s		
none	0.02 ± 0.01	0.02 ± 0.01
0.5 nM α-thrombin	0.30 ± 0.28	0.06 ± 0.03
1.0 nM α-thrombin	0.85 ± 0.34	0.10 ± 0.03
10 μM ADP + 40 μg/mL fibrinogen	0.11 ± 0.03	0.14 ± 0.06
100 μM arachidonate	0.06 ± 0.02	0.04 ± 0.01
2 μg/mL collagen	0.04 ± 0.01	0.03 ± 0.01
10 μM FSLLRN	0.03 ± 0.02	0.004 ± 0.004
100 μM YAPGKF	0.06 ± 0.02	0.02 ± 0.01

[a] Washed human platelets were resuspended in buffer (5×10^{11}/L) and incubated in the presence or absence of agonist. Aliquots were removed at 10 s (A), 60 s (not shown), and 180 s (B), and [PAR-1-(1−41)] and [PAR-4-(1−47)] were measured in the platelet supernatants by specific ELISAs. Data are presented as mean ± SEM for three to seven different platelet preparations.

platelets were preincubated with inhibitors as described. Aliquots (190 μL) of washed platelets (5×10^{11}/L) loaded with 10 μM Fura-PE3 (AM) were added in duplicate to black 96-well plates. Using the Fluoroskan Ascent microplate fluorometer (Thermo Labsystems, Thermo Electron Corp., Milford, MA), the Fura-PE3 (AM) labeled platelets were subjected to an excitation wavelength of 340 nm while measuring emission at 510 nm. The extracellular [$CaCl_2$] was 2 mM. The intensity of fluorescence was measured for 120 s after addition of 10 μL of agonist. After each experiment the platelets were treated with 0.2% Triton X-100 followed by the addition of 10 mM EGTA to obtain the maximum and minimum fluorescence, respectively. The [Ca^{2+}] was calculated according to the equation $[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F)$ (*24*) using a dissociation constant for Fura-PE3 (AM) and Ca^{2+} of 264 nM. Data are expressed as a percent of control using the total area under the curve.

Measurement of ATP Release. Washed platelets (5×10^{11}/L) were stimulated with 0.5 or 1.0 nM α-thrombin, 10 μM SFLLRN, or 100 μM AYPGKF without stirring. After 180 s, aliquots (50 μL) were added to a solution containing 1 μM FPR chloromethyl ketone, 1 μM 1,5-dansyl-EGR chloromethyl ketone (Calbiochem, La Jolla, CA), and 6.25 mM EDTA and mixed well. The samples were then centrifuged for 2 min at 10000*g* and the supernatants removed. ATP levels in the supernatants were determined in duplicate using the ATP determination kit (A-22066; Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Luminescence of the samples was measured using a Tropix TR717 microplate luminometer (Perkin-Elmer Applied Biosystems, Foster City, CA). The [ATP] in the experimental samples were interpolated from a standard curve. The effect of each agent on the [ATP] measured in the absence of platelets was determined to be insignificant (data not shown).

Density of PAR-1 on Platelets Detected with WEDE 15. Washed platelet preparations were incubated in the absence or presence of 1.0 nM α-thrombin, 10 μM SFLLRN, or 100 μM AYPGKF without stirring for 180 s, and 5 mM EDTA and 1 μM hirudin (Berlex, Canada, Inc., Pointe Claire, Quebec, Canada) were then added. The platelets were divided and incubated for 1 h with buffer alone or 1 μg/mL PE-conjugated WEDE 15 (Immunotech, Marseille, France). The expression of PAR-1 on the surfaces of 10000 platelets was quantified by flow cytometry, as previously described (*1*).

Data Analysis. The data were analyzed using GraphPad Prism version 4.02 for Windows, GraphPad Software (San Diego, CA).

RESULTS

PAR-1-(1−41) and PAR-4-(1−47) Are Generated Rapidly in Response to Nonproteolytic Platelet Agonists. Washed human platelets incubated with 10 μM SFLLRN, 100 μM AYPGKF, 500 μM arachidonic acid, or 0.5 nM (not shown) or 1.0 nM α-thrombin (not shown) generate simultaneously PAR-1-(1−41) and PAR-4-(1−47) that are detected in the platelet supernatants within 10 s of each agonist addition (Table 1A). The number of PAR-1 molecules per platelet is ~1800 (*25*). The maximum [PAR-1-(1−41)] that could be generated is ~1.5 nM. Generation of PAR-1-(1−41) and PAR-4-(1−47) is complete 10 s after the addition of 10 μM SFLLRN or 100 μM AYPGKF or 500 μM arachidonate (Table 1A), while it only approaches completion 180 s after the addition of 1.0 nM α-thrombin (Table 1B). The scrambled PAR-1 and -4 tethered ligand peptides, FSLLRN (10 μM) and YAPGKF (100 μM), elicited no PAR peptide release (Table 1B) nor any other platelet aggregation-related responses (not shown). Incubation of platelets with collagen, ADP plus fibrinogen, or arachidonic acid resulted in the expected platelet aggregation. However, less PAR-1-(1−41) and PAR-4-(1−47) were generated even after 180 s in response to collagen or ADP plus fibrinogen (Table 1B). As noted previously, PAR-1-(1−41) and PAR-4-(1−47) are generated within 10 s, and prior to the initiation of platelet aggregation, after platelets are incubated with α-thrombin, SFLLRN, AYPGKF, or 500 μM arachidonic acid. In contrast, both peptides are first detected at 180 s after platelets are incubated with ADP plus fibrinogen or with collagen, i.e., ≥150 s after the onset of platelet aggregation. Therefore, not all platelet agonists rapidly elicit the generation of PAR-1-(1−41) and PAR-4-(1−47) prior to the initiation of platelet aggregation.

Contributions of PAR-1 and -4 Signaling to the Development of Platelet Aggregation-Related Responses to α-Thrombin. We compared the effects of BMS 200261 (a specific SFLLRN and thus PAR-1 antagonist (*4*)) and YD3 (a specific PAR-4 antagonist (*5*)) on PAR-1-(1−41) and PAR-4-(1−47) generation and the subsequent aggregation-related responses.

Incubation of platelets with 1 μM BMS 200261 before SFLLRN decreased generation of the PAR-1 and -4 amino-terminal peptides by ≥70% and completely prevented ATP release and platelet aggregation. In contrast, BMS 200261 enhanced PAR-1-(1−41) and PAR-4-(1−47) generation in response to AYPGKF by ≥20% and had no effect on ATP release or platelet aggregation. Preincubation of platelets with BMS 200261 before 1.0 nM α-thrombin decreased generation of the two peptides by approximately 50%, ATP secretion by 24%, and aggregation by 32%. Thus, although BMS 200261 prevents exogenous SFLLRN binding to PAR-1 (*4*) and may also prevent the binding of endogenously generated PAR-1 tethered ligand to PAR-1, PAR-4-(1−47) is generated, ATP is released, and the platelets aggregate, in response to 1.0 nM α-thrombin. The inhibitory effects of SCH79797 (1 μM) on platelet responses to SFLLRN or α-thrombin were similar to those of BMS 200261 (not shown).

PAR-4 inhibition with 2.8 μM YD3 prevented platelets from aggregating or releasing ATP in response to AYPGKF, as expected (*2, 5*), even though YD3 inhibited PAR-1-(1−41) and PAR-4-(1−47) generation by only 60%. In contrast, YD3 did not inhibit any platelet aggregation-related responses to SFLLRN. The only platelet response to 1.0 nM α-thrombin affected by YD3 was ATP release (∼20% inhibition). Thus, specific PAR-4 inhibition had relatively little effect on most platelet responses to 1.0 nM α-thrombin. *trans*-Cinnamoyl-YPGKF, a second PAR-4 antagonist (*26*), had no effect on the responses of human platelets to AYPGKF, SFLLRN, or α-thrombin. This agent has thus far been shown to inhibit PAR-4 in rat platelets (*27*).

A combination of BMS 200261 and YD3 eliminated all platelet responses to 1.0 nM α-thrombin. Neither BMS 200261 nor YD3 inhibited platelet aggregation initiated with 500 μM arachidonic acid, but a combination of the two PAR antagonists inhibited platelet aggregation by ∼30% without inhibiting the generation of PAR-1-(1−41) and PAR-4-(1−47) (not shown). A combination of SCH79797 and YD3 also inhibited platelet aggregation in response to 500 μM arachidonate by ∼20% but had no effect on PAR-1-(1−41) and PAR-4-(1−47) generation (not shown). Platelets pretreated with aspirin or PGE_1 did not respond to 500 μM arachidonic acid. Thus, platelet signaling initiated with 500 μM arachidonic acid (i.e., via phospholipase A_2−thromboxane A_2 axis) results in rapid generation of the platelet proteinase that liberates PAR-1-(1−41) and PAR-4-(1−47). However, the inability of the combination of PAR-1 and -4 antagonists to effectively suppress platelet responses to 500 μM arachidonate suggests that the concentration of thromboxane A_2 generated *in situ* from the added 500 μM arachidonate is sufficient to activate the platelets and that the endogenously generated PAR-1 and -4 tethered ligands do not make major contributions to the elaboration of the arachidonate-initiated platelet responses observed. Consistent with this idea, both 100 and 500 μM arachidonate equally and effectively activate washed platelets. However, only ≤0.06 nM PAR-1-(1−41) or PAR-4-(1−47) is generated 180 s after platelets are incubated to 100 μM arachidonate (Table 1). The low concentrations of PAR-1-(1−41) and PAR-4-(1−47) generated at 180 s in response to 100 μM arachidonate explain why the two combinations of PAR-1 and PAR-4 antagonists do not effectively inhibit arachidonate-initiated platelet aggregation. Thus, generation of PAR-1 and -4 tethered ligands *in situ* (indirectly estimated from the liberation of PAR-1-(1−41) or PAR-4-(1−47) respectively) is not required to drive platelet aggregation-related responses to arachidonic acid. Because SBTI eliminates all platelet responses to arachidonate, the platelet serine protease that generates PAR-1-(1−41) or PAR-4-(1−47) probably catalyzes another early but unknown reaction critical for platelet activation in addition to generating these two PAR-derived peptides.

The Platelet-Derived Serine Proteinase Acts within 10 s after Platelets Are Incubated with α-Thrombin, SFLLRN, or AYPGKF. As observed previously, preincubation of platelets with SBTI prevented PAR-1-(1−41) generation and CD62 expression on platelets in response to α-thrombin or SFLLRN (*1*). SBTI also prevented all platelet aggregation-related responses to AYPGKF in this study, including the generation of PAR-1-(1−41) or PAR-4-(1−47). Completion of PAR-1-(1−41) and PAR-4-(1−47) generation 10 s following the addition of SFLLRN, AYPGKF, or 500 μM arachidonic acid to control platelets, compared to the slower rates associated with their generation in response to 0.5 and 1.0 nM α-thrombin, led us to compare how SBTI influences platelet aggregation if it was added to platelets before and 5 or 10 s after each agonist addition. Essentially no platelet aggregation was observed after 0.5 or 1.0 nM α-thrombin, SFLLRN, AYPGKF, or arachidonic acid was added to platelets that had been preincubated with SBTI (Figure 1). Furthermore, neither PAR-1-(1−41) nor PAR-4-(1−47) was generated when platelets were preincubated with SBTI prior to any one of the three agonists. However, if the platelets were first incubated with 1.0 nM α-thrombin, 10 μM SFLLRN, or 100 μM AYPGKF for 10 s before SBTI addition, aggregation was less effectively inhibited. In contrast, platelets incubated with 0.5 nM α-thrombin or 500 μM arachidonic acid for 10 s before SBTI addition failed to aggregate. In additional experiments, SBTI did not prevent aggregation of platelets preincubated with 1.0 nM α-thrombin for 5 s prior to SBTI (Figure 1). Platelets preincubated with SFLLRN for 5 s prior to SBTI addition aggregated partially, but the aggregation was rapidly reversible. Platelets preincubated for 5 s with 0.5 nM α-thrombin, AYPGKF, or arachidonic acid before SBTI failed to aggregate. These results support an active and early role (i.e., within 10 s after agonist addition) for a platelet-derived trypsin-like proteinase in optimizing platelet aggregation-related responses. Platelet aggregation to arachidonic acid was abrogated by prior treatment of the washed platelets with aspirin, as expected (not shown). In contrast, aspirin-treated platelets aggregated normally in response to α-thrombin, SFLLRN, or AYPGKF (not shown).

Elevation of Intraplatelet [cAMP] Eliminates PAR-1-(1−41) and PAR-4-(1−47) Generation and Other Platelet Responses. The cell signaling inhibitors had different effects on the measured platelet responses to α-thrombin (Table 2), SFLLRN (Table 3), and AYPGKF (Table 4). PAR-1-(1−41) and PAR-4-(1−47) generation, expression of CD62, ATP, and [^{14}C]serotonin release, intraplatelet Ca^{2+} mobilization, and aggregation were measured as indices of platelet responses to the agonists.

Normally, after G_i-coupled thrombin receptors on platelets bind their physiological ligands, intracellular [cAMP] decreases and both ADP and ATP are released (*9*−*11*). PGE_1

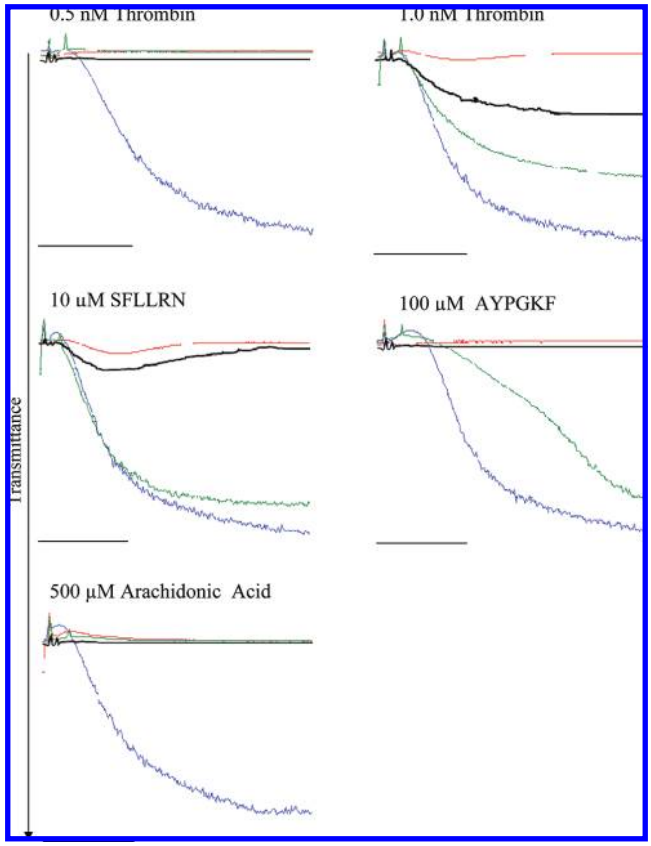

FIGURE 1: SBTI inhibits aggregation initiated by 0.5 or 1 nM α-thrombin, 10 μM SFLLRN, 100 μM AYPGKF, or 500 μM arachidonic acid but has less effect when added 10 s after the agonist. Washed human platelets were resuspended in buffer (5×10^{11}/L) and incubated for <60 s in the absence (blue) or presence (red) of 10 μM SBTI before agonist addition. Alternatively, SBTI was added 5 s (black) or 10 s (green) after agonist addition. Aggregation was observed for 3 min with constant stirring. The data shown are representative of at least four platelet preparations. The horizontal line under each set of tracings represents 1 min.

and forskolin eliminated not only ATP release but also nearly prevented PAR-1-(1−41) and PAR-4-(1−47) generation in response to 1.0 nM α-thrombin (Table 2) and 10 μM SFLLRN (Table 3). With the exception of intraplatelet Ca^{2+} mobilization, PGE_1 eliminated or nearly eliminated all platelet responses to α-thrombin or SFLLRN. Forskolin also abrogated or nearly abrogated all platelet responses to the two agonists. Forskolin eliminated all platelet responses to AYPGKF (Table 4). While PGE_1 did not entirely prevent the generation of PAR-1-(1−41) and PAR-4-(1−47) in response to AYPGKF, it eliminated all the other platelet responses to this PAR-4-dependent agonist, with the exception of intraplatelet Ca^{2+} mobilization (Table 4). Therefore, the previously reported agonist-induced decreases in intraplatelet [cAMP] and release of ATP (*9*−*11*) may precede effective PAR-1-(1−41) and PAR-4-(1−47) generation and most of the subsequent platelet aggregation-related responses to PAR-1-dependent and PAR-4-dependent agonists.

PKC Inhibition Reduces PAR-1-(1−41) and PAR-4-(1−47) Generation and Other Platelet Responses. BIMI-1 reduced PAR-1-(1−41) and PAR-4-(1−47) generation in response to α-thrombin to about 50% of control (Table 2). The SFLLRN- and AYPGKF-initiated generation of PAR-1-(1−41) and PAR-4-(1−47) was inhibited slightly more effectively than α-thrombin-initiated generation of these two peptides, but ATP release and platelet aggregation in response to each of the three agonist were almost completely inhibited (Tables 2−4). In the presence of this PKC inhibitor, increased calcium mobilization in response to α-thrombin was observed (Table 2), as has been previously reported (*28*), and to AYPGKF (Table 4). However, BIMI-1 had no effect on SFLLRN-initiated in intracellular calcium mobilization (Table 3). Therefore, the increase in calcium mobilization observed with 1 nM α-thrombin probably arises from PAR-4-dependent signaling. Subsequent PKC-catalyzed reactions directly or indirectly accelerate PAR-1 and -4 activation, platelet secretion, and aggregation. Whether these agonist-induced increases in intracellular [Ca^{2+}] accelerated PAR-1 and -4 activation and CD62 expression that probably occur prior to PKC activation is not known.

Inhibition of PLC or PI3-Kinase Does Not Reduce PAR-1-(1−41) and PAR-4-(1−47) Generation but Downregulates Subsequent Platelet Responses. Inhibition of PLC with U73122 did not reduce PAR-1-(1−41) and PAR-4-(1−47) generation in response to α-thrombin (Table 2). However, all of the subsequent platelet responses to α-thrombin, except intraplatelet Ca^{2+} mobilization, were effectively inhibited. SFLLRN-initiated PAR-1-(1−41) generation was slightly inhibited by U73122, while PAR-4-(1−47) generation was not. Despite its inability to suppress generation of the two peptides, all of the other platelet activation indices, except intraplatelet Ca^{2+} mobilization, were markedly decreased by PLC inhibition (Table 3). With the exception of ATP release, AYPGKF-initiated platelet responses were not decreased by PLC inhibition (Table 4). Therefore, PAR-1 and -4 amino-peptide generation in response to α-thrombin or AYPGKF is independent of PLC. However, PLC may be required for optimal PAR-1-(1−41) generation when initiated by SFLLRN. PLC is required for optimal platelet aggregation responses to α-thrombin and SFLLRN.

Inhibition of PI3-kinase with LY294002 did not significantly decrease PAR-1-(1−41) and PAR-4-(1−47) release in response to α-thrombin (Table 2), SFLLRN (Table 3), or AYPGKF (Table 4). However, some platelet responses to both α-thrombin and SFLLRN, but not to AYPGKF, were decreased by PI3-kinase inhibition with LY294002. Therefore, optimal PAR-1-(1−41) and PAR-4-(1−47) generation, which is an ATP-dependent reaction, probably precedes PI3-kinase activation. LY294002 inhibited changes in intracellular calcium concentration initiated by SFLLRN and AYPGKF signaling but had no effect on thrombin-initiated changes in intracellular calcium concentration.

Density of PAR-1 on Platelets Detected with WEDE 15. Cleavage of PAR-1 at any site downstream of $R^{41}S^{42}$ would disable PAR-1 since all of the first six residues of the PAR-1-tethered ligand domain, namely, S^{42}FLLRN, contribute to PAR-1-dependent signaling that occurs after PAR-1 is activated (*29*). Fragments of the first extracellular domain of PAR-1 larger than PAR-1-(1−41) that would disable PAR-1 are probably undetectable by the ELISA that measures PAR-1-(1−41) generation. We attempted to rule out the generation of such larger PAR-1 fragment peptides by measuring, using flow cytometry, the percent platelets that bound WEDE 15, a monoclonal antibody directed against the hirudin-like domain, PAR-1-(51−64) (*25*). Approximately 35% of control platelets, and platelets activated with α-thrombin or SFLLRN, bound WEDE 15. In contrast,

Table 2: Effects of Signaling Inhibitors on Platelet Responses to 1.0 nM α-Thrombin[a]

		% control (mean ± SEM)						
target	agent	[PAR-1-(1−41)], 60 s	[PAR-4-(1−47)], 60 s	ATP release	[^{14}C]serotonin release	CD62 expression	aggregation	[Ca^{2+}]$_i$, 120 s
AC	PGE_1	18 ± 6[b]	18 ± 5[b]	0.2 ± 0.1[b]	11 ± 7[b]	10 ± 2[b]	8 ± 8[b]	41 ± 14[b]
AC	forskolin	11 ± 3[b]	20 ± 6[b]	0 ± 0[b]	3 ± 0.4[b]	7 ± 2[b]	0 ± 0[b]	19 ± 3[b]
PKC	BIMI-1	47 ± 8[b]	54 ± 8[b]	1 ± 1[b]	not done	70 ± 13	16 ± 7[b]	323 ± 86
PLC	U73122	97 ± 9	109 ± 12	46 ± 8[b]	10 ± 3[b]	41 ± 10[b]	15 ± 6[b]	103 ± 13
PI3-K	LY294002	94 ± 8	86 ± 11	63 ± 7[b]	not done	71 ± 10	72 ± 9[b]	127 ± 13

[a] Washed human platelets were resuspended in buffer (5×10^{11}/L), incubated for 30 min in the presence or absence (control) of 10 μM PGE_1, 10 μM forskolin, 5 μM BIMI-1, 20 μM U73122, or 12.5 μM LY294002, and then activated with 1 nM α-thrombin. Aliquots were removed at 10 s (not shown), 60 s, and 180 s (not shown), and [PAR-1-(1−41)] and [PAR-4-(1−47)] were measured in the platelet supernatants by specific ELISAs. [ATP] released into the platelet supernatants was measured after 180 s. Aliquots were removed and fixed at 180 s, and the percentages of platelets expressing CD62 and CD63 (not shown) were determined by flow cytometry. [^{14}C]Serotonin release and platelet aggregation were measured 180 s after addition of α-thrombin. Changes in intracellular [Ca^{2+}] were measured fluorometrically for 120 s after the addition of α-thrombin, and the area under the curve was calculated. The data are expressed as % of control (mean ± SEM) for at least four platelet preparations. [b] Indicates $p < 0.05$ using one-sample *t* test to compare to a hypothetical mean of 100%.

Table 3: Effects of Signaling Inhibitors on Platelet Responses to 10 μM SFLLRN[a]

		% control (mean ± SEM)						
target	agent	[PAR-1-(1−41)], 60 s	[PAR-4-(1−47)], 60 s	ATP release	[^{14}C]serotonin release	CD62 expression	aggregation	[Ca^{2+}]$_i$, 120 s
AC	PGE_1	19 ± 8[b]	16 ± 6[b]	0 ± 0[b]	9 ± 7[b]	15 ± 8[b]	10 ± 10[b]	78 ± 14
AC	forskolin	7 ± 2[b]	19 ± 8[b]	0.1 ± 0.1[b]	3 ± 1[b]	11 ± 9[b]	0 ± 0[b]	3 ± 7[b]
PKC	BIMI-1	37 ± 8[b]	36 ± 9[b]	0 ± 0[b]	not done	51 ± 13[b]	8 ± 6[b]	82 ± 16
PLC	U73122	73 ± 12[b]	100 ± 11	15 ± 7[b]	20 ± 8[b]	19 ± 6[b]	20 ± 9[b]	76 ± 16
PI3-K	LY294002	103 ± 4	94 ± 7	52 ± 11[b]	not done	36 ± 3[b]	64 ± 10[b]	59 ± 5[b]

[a] Washed human platelets were resuspended in buffer (5×10^{11}/L), incubated for 30 min in the presence or absence (control) of 10 μM PGE_1, 10 μM forskolin, 5 μM BIMI-1, 20 μM U73122, or 12.5 μM LY294002, and then activated with 10 μM SFLLRN. Aliquots were removed at 10 s (not shown), 60 s, and 180 s (not shown), and [PAR-1-(1−41)] and [PAR-4-(1−47)] were measured in the platelet supernatants by specific ELISAs. [ATP] released into the platelet supernatants was measured after 180 s. Aliquots were removed and fixed at 180 s, and the percentages of platelets expressing CD62 and CD63 (not shown) were determined by flow cytometry. [^{14}C]Serotonin release and platelet aggregation were measured 180 s after addition of SFLLRN. Changes in intracellular [Ca^{2+}] were measured fluorometrically for 120 s after the addition of SFLLRN, and the area under the curve was calculated. Note that generation of PAR-1-(1−41) and PAR-4-(1−47) was essentially complete 10 s after SFLLRN was added to control platelets. Thus, PGE_1, forskolin, and BIMI-1 very effectively inhibited the generation of PAR-1-(1−41) and PAR-4-(1−47) and platelet aggregation in response to SFLLRN. The data are expressed as % of control (mean ± SEM) for at least four platelet preparations. [b] Indicates $p < 0.05$ using one-sample *t* test to compare to a hypothetical mean of 100%.

Table 4: Effects of Signaling Inhibitors on Platelet Responses to 100 μM AYPGKF[a]

		% control (mean ± SEM)					
target	agent	[PAR-1 (1−41)], 60 s	[PAR-4-(1−47)], 60 s	ATP release	CD62 expression	aggregation	[Ca^{2+}]$_i$, 120 s
AC	PGE_1	28 ± 20[b]	54 ± 20	3 ± 3[b]	13 ± 5[b]	7 ± 7[b]	56 ± 29
AC	forskolin	2 ± 1[b]	5 ± 2[b]	0 ± 0[b]	12 ± 4[b]	0 ± 0[b]	18 ± 6[b]
PKC	BIMI-1	23 ± 13[b]	42 ± 14[b]	0 ± 0[b]	48 ± 15[b]	0 ± 0[b]	195 ± 71
PLC	U73122	94 ± 15	130 ± 27	48 ± 16[b]	81 ± 8	78 ± 12	68 ± 15
PI3-K	LY294002	131 ± 20	97 ± 9	74 ± 38	96 ± 2	90 ± 8	44 ± 10[b]

[a] Washed human platelets were resuspended in buffer (5×10^{11}/L), incubated for 30 min in the presence or absence (control) of 10 μM PGE_1, 10 μM forskolin, 5 μM BIMI-1, 20 μM U73122, or 12.5 μM LY294002, and then activated with 100 μM AYPGKF. Aliquots were removed at 10 s (not shown), 60 s, and 180 s (not shown), and [PAR-1-(1−41)] and [PAR-4-(1−47)] were measured in the platelet supernatants by specific ELISAs. [ATP] released into the platelet supernatants was measured after 180 s. Aliquots were removed and fixed at 180 s, and the percentages of platelets expressing CD62 and CD63 (not shown) were determined by flow cytometry. Changes in intracellular [Ca^{2+}] were measured fluorometrically for 120 s after the addition of AYPGKF, and the area under the curve was calculated. Note that generation of PAR-1-(1−41) and PAR-4-(1−47) was essentially complete 10 s after AYPGKF was added to control platelets. Thus, PGE_1 and forskolin very effectively inhibited the generation of PAR-1-(1−41) and PAR-4-(1−47) and platelet aggregation in response to AYPGKF. The data are expressed as % of control (mean ± SEM) for at least four platelet preparations. [b] Indicates $p < 0.05$ using one-sample *t* test to compare to a hypothetical mean of 100%.

WEDE 15 bound to ~70% of platelets activated with AYPGKF. Therefore, we found no evidence that release of PAR-1-(1−41) or other fragments detected by the ELISA for PAR-1-(1−41) from the first extracellular domain of PAR-1 in response to α-thrombin destroyed the PAR-1 epitope that binds WEDE 15. Furthermore, we found no evidence consistent with only disabling cleavages of PAR-1 since essentially complete liberation of PAR-1-(1−41) 10 s after control-washed platelets were incubated with either SFLLRN or AYPGKF (Table 1) was associated with normal platelet aggregation (Tables 2−4). Liberation of a similar concentration of PAR-1-(1−41) at 180 s in response to 1 nM α-thrombin was also associated with normal platelet aggregation-related responses. In contrast, prevention of agonist-mediated platelet aggregation-related responses with forskolin, for example, was associated with ≥80% inhibition of PAR-1-(1−41) and PAR-4-(1−47) generation (Tables 2−4).

DISCUSSION

PAR-1 activation has several consequences on human platelet physiology (*30, 31*). PAR-1 activation resulting from

its cleavage of $R^{41}S^{42}$ unmasks the tethered ligand of the receptor to propagate PAR-1 activation (*30, 32*−*34*) as well as PAR-4 activation (ref *2* and this study). PAR-1 and -4 activation may be estimated as the release of PAR-1-(1−41) and PAR-4-(1−47) into the platelet supernatants in response to ≤1.0 nM α-thrombin, SFLLRN, or AYPGKF (*1, 2, 18*). Matrix metalloprotease-1 activates wild-type PAR-1 (*35*), as does cathepsin G (*36*). In heterologous cell expression systems, replacement of the thrombin cleavage site of PAR-1 with a trypsin or an enterokinase cleavage site allows for trypsin- or enterokinase-mediated cell signaling (*37*). Therefore, proteinases other than α-thrombin can effectively cleave PAR-1 at $R^{41}S^{42}$ to potentially unmask the previously cryptic PAR-1 tethered ligand. It is also conceivable that other proteinases can activate PAR-4 at $R^{47}G^{48}$ to thereby unmask its tethered ligand and initiate and propagate PAR-4-dependent cell signaling. Thereafter, the ATP released binds to its receptors and couples with members of $G_{12/13}$, G_q, and $G_{i/2}$ families of G-proteins, to accelerate platelet activation (*31, 38, 39*).

Early platelet activation signaling events in response to α-thrombin or SFLLRN, the first six residues of the tethered ligand domain of PAR-1, include agonist stimulation of G-protein-coupled receptors, inhibition of adenylyl cyclase (*30*), and the rapid stimulation of phospholipase Cβ (PLCβ) by the agonist-activated G-proteins (*40*). PLCβ catalyzes the ATP-dependent rapid hydrolysis of PIP_2 into PIP_3 (IP_3) and diacylglycerol (DAG) (*41*). IP_3 causes the influx of Ca^{2+} into the cytoplasm after it binds to its receptors in the dense tubular system and cell membranes (*15, 40, 41*). DAG activates protein kinase C (PKC) in a Ca^{2+}- and ATP-dependent manner (*39*−*44*). Thus, agonist-induced increase in platelet cytosolic [Ca^{2+}] facilitates PKC activation.

ATP binding to the P2X1 receptor on platelets is another mode for rapidly increasing Ca^{2+} influx into the platelet cytosol, as well as inducing platelet shape change (*42, 45*−*50*). Thus, ATP can reasonably be considered to be a significant secondary platelet agonist in a similar way as epinephrine, platelet-derived ADP, and thromboxane A2. Phosphoinositol 3-kinase activated by Gβγ subunits is another important regulator of platelet responses to α-thrombin (*31, 38, 40, 45*−*50*). Phosphoinositol 3-kinases catalyze ATP-dependent phosphorylation of the D3 hydroxyl group of phosphoinositides involved in calcium signaling, cytoskeleton rearrangement, GPIIb/IIIa activation, and the irreversible phase of platelet activation (*31, 40, 43, 51*). Ligand occupancy of the platelet ADP receptor linked to G_i, or PAR-1 linked to G_i, inhibits adenylyl cyclase to suppress cAMP levels (*31, 43*). Suppression of cAMP levels is an early and critical intracellular event during agonist-initiated platelet activation. Specifically, this potentiates ADP-induced aggregation via $P2Y_{12}$, and thrombin-induced aggregation via PAR-1, and this is augmented by the ADP released from dense granules binding to $P2Y_{12}$ (*11, 31, 42, 43, 52*−*58*). PI3-kinase inhibition decreases changes in intracellular [Ca^{2+}] and prevents granular ATP secretion and P-selectin expression (*43*). $P2Y_1$, the second platelet receptor for ADP that is linked to G_q, activates PLC to mobilize Ca^{2+} from the intracellular stores to activate PKC (*31, 43*). Occupancy of the ADP receptors and $P2X_1$ by the ADP and ATP released from the platelet dense granules enhances platelet responses to α-thrombin (*31, 42, 43*). Elevation of intraplatelet cAMP levels with PGE_1 or forskolin decreases the turnover of phosphoinositides and thrombin-mediated secretion from human platelets (*9, 10, 41*). With the above background information, the effects of the platelet antagonists on activation of PAR-1 and -4 and their propagation in response to α-thrombin can be given some perspective.

As detailed in this and a previous study (*2*), simultaneous generation of PAR-1-(1−41) and PAR-4-(1−47), and measurable within 10 s of agonist addition, precedes platelet aggregation in response to α-thrombin. This early and simultaneous generation of the two peptides may be attributable, at least in part, to the presence of stable heterodimers of PAR-1 and -4 on human platelets (*59*). Significantly, both PAR-1-(1−41) and PAR-4-(1−47) are generated more rapidly in response to SFLLRN or AYPGKF than α-thrombin. Therefore, agonist-induced exposures of the previously cryptic endogenous tethered ligand domains of PAR-1 and -4 (and their ligation of their respective PARs *30, 32*−*34*) probably contribute to the rapid generation of the platelet-derived proteinase that propagates PAR-1-(1−41) and PAR-4-(1−47) generation. Previous studies had demonstrated that PAR-1-(1−41), and not truncated versions of this peptide (i.e., PAR-1-(1−21) or PAR-1-(21−41)) or extruded PAR-1, is generated after platelets are incubated with α-thrombin or SFLLRN (*1, 60*). This study explored whether fragments of the first extracellular domain of PAR-1 larger than PAR-1-(1−41) are also generated after platelets are incubated with α-thrombin, SFLLRN, or AYPGKF. Exclusive generation of fragments of PAR-1 larger than PAR-1-(1−41) would probably eliminate PAR-1-dependent signaling normally propagated by the PAR-1-tethered ligand domain. Mass spectrometry is the definitive tool for establishing the mass of the peptides generated after the first extracellular domains of PAR-1 and -4 are cleaved in response to α-thrombin, SFLLRN, or AYPGKF. In collaboration with the Mass Spectrometry Groups at the Department of Chemistry at McMaster University and Toronto General Hospital, University of Toronto, attempts were made to determine the mass of the peptides that were routinely quantified in this study using the specific ELISAs for measuring PAR-1-(1−41) and PAR-4-(1−47). Due to the low (subnanomolar) concentrations of the peptides generated from platelets together with the many other proteins that platelets routinely release during their activation (*23*), the mass of the PAR-1-(1−41)- and PAR-1-(1−47)-related peptides generated and quantified by their respective ELISAs could not be determined using mass spectrometry.

The monoclonal antibody (WEDE 15) directed against the hirudin-like domain of PAR-1 (i.e., PAR-1-(51−64)) that is downstream of PAR-1 $R^{41}S^{42}$ binds both resting and activated platelets (*25*). Incubation of platelets with α-thrombin or SFLLRN did not alter the percentage of platelets that bound this monoclonal antibody, as estimated by flow cytometry (35%). Surprisingly, the percentage of platelets that bound WEDE 15 doubled after platelets were incubated with AYPGKF. A monoclonal anti-PAR-4 antibody that binds to residues in the first extracellular domain of PAR-4 downstream from PAR-4-(1−47) is currently unavailable. We therefore could not determine whether cleavages of PAR-4 that would disable this receptor occur after platelets were incubated with α-thrombin, SFLLRN, or AYPGKF. Thus, our results do not definitively rule out SFLLRN- or AYPGKF-dependent generation of fragments larger than PAR-1-(1−41) and/or PAR-4-(1−47) that would disable PAR-1-

and -4-dependent signaling. Similarly, generation of only PAR-1-(1−41) and PAR-4-(1−47) in response to α-thrombin could be considered to be tentative. Initiation of aggregation-related responses of platelets to α-thrombin has been attributable to the generation *in situ* of the endogenous tethered ligand domains of PAR-1 and -4 (*30, 53, 61*). Furthermore, we have previously reported that a combination of chicken anti-human PAR-1-(35−62)−IgY and anti-human PAR-4-(34−54)−IgY prevents both PAR-1-(1−41) and PAR-4-(1−47) generation in response to α-thrombin and simultaneously prevents platelet aggregation and release. Given that PAR-1-(1−41) and PAR-1-(35−62) share common residues (i.e., PAR-1-(35−41)) and that PAR-4-(1−47) and PAR-4-(34−54) also share common residues (i.e., PAR-4-(34−47)), these results could be interpreted to indicate that anti-PAR-1-(35−62)−IgY and anti-PAR-4-(34−54)−IgY prevent the detection of PAR-1-(1−41) and PAR-4-(1−47), respectively, by ELISA. However, the combination of anti-PAR-1-(35−62)−IgY and anti-PAR-4-(34−54)−-IgY has no effect on the quantification of PAR-1-(1−41) or PAR-4-(1−47) generated by platelets incubated with SFLLRN or AYPGKF (*2*). These results provide additional indirect evidence that amino-terminal fragments derived from the first extracellular domains of PAR-1and PAR-4 that result from cleavages downstream of PAR-1 R^{41} and PAR-4 R^{47}, respectively, are unlikely to be generated during platelet responses to α-thrombin, SFLLRN, or AYPGKF. These results also suggest that some cleavage of PAR-1 at $R^{41}S^{42}$ and PAR-4 at $R^{47}G^{48}$ clearly must have occurred to account for at least some of the PAR-1-(1−41) and PAR-4-(1−47) measured and for the aggregation-related responses of washed platelets to SFLLRN or AYPGKF that were observed. Both PAR-1-(1−41) and PAR-4-(1−47) must clearly have been generated after platelets were activated in response to α-thrombin (*30, 53, 61*).

Platelet activation-related responses to SFLLRN or α-thrombin are inhibited by soybean trypsin inhibitor (*1*). As shown in this study, platelet aggregation-related responses to AYPGKF or arachidonic acid are also inhibited by SBTI. Aprotinin, a trypsin-like proteinase inhibitor (excluding α-thrombin), reduces PAR-1-dependent platelet activation during cardiopulmonary bypass grafting and *in vitro* (*6, 7, 62, 63*). Therefore, PAR-1-(1−41) and PAR-4-(1−47) generation in response to SFLLRN, AYPGKF, or α-thrombin is propagated by a platelet-derived serine proteinase that is inactivated by these two Kunitz-type inhibitors of trypsin-like enzymes. Activation of this platelet-derived proteinase in response to 1 nM α- thrombin, 10 μM SFLLRN, or 100 μM AYPGKF can be demonstrated within 10 s of agonist addition to platelets. Specifically, generation of PAR-1-(1−41) and PAR-4-(1−47) by the platelet-derived proteinase within 10 s has proceeded to the extent that SBTI no longer prevents the aggregation of platelets that have been preincubated with the above concentrations of α- thrombin, SFLLRN, or AYPGKF for 10 s (Figure 1). While matrix metalloproteinase-2 (MM-2) has a role in platelet responses to collagen, thrombin, or SFLLRN (*64, 65*), this platelet metalloproteinase may have no role in the generation of PAR-1-(1−41) and PAR-4-(1−47). Specifically, we previously reported that two specific inhibitors of metalloproteinases (*o*-phenanthroline or phosphoramidon), as well as specific inhibitors of calpain, chymotrypsin, or factor Xa do not alter PAR-1-(1−41) generation or the other responses of washed platelets to SFLLRN or α-thrombin (*1*). It is noteworthy that up to 1.0 ng/mL recombinant MM-2, but not pro-MM-2, enhances platelet responses to collagen or thrombin (*64, 65*). Platelets stimulated with SFLLRN for 4 min do express surface pro-MM-2, but not MM-2. Furthermore, conversion of pro-MM-2 expressed on SFLLRN-stimulated platelets to MM-2 is first observed 30 min after the platelets have been stimulated with SFLLRN (*64*). As noted previously (*2*), generation of PAR-1-(1−41) and PAR-4-(1−47) is complete within 10 s after incubating platelets with SFLLRN or AYPGKF. Based on these considerations, platelet MM-2 is unlikely to contribute significantly to PAR-1-(1−41) and PAR-4-(1−47) generation during SFLLRN- or AYPGKF-mediated platelet activation normally quantified for only 3 min.

The major goal of this study was to identify the intraplatelet signaling pathways that directly or indirectly cause the platelet-derived serine proteinase responsible for cleaving PAR-1 and -4 to be generated. A secondary goal was to determine whether there were conditions under which optimal generation of PAR-1-(1−41) and PAR-4-(1−47) was observed without significant propagation of the other platelet responses to α-thrombin, SFLLRN, or AYPGKF. The results provide insights on the signaling reactions in platelets that likely influence PAR-1-(1−41) and PAR-4-(1−47) generation in response to these agonists.

Forskolin and PGE_1, which elevate intraplatelet concentrations of cyclic AMP (*9−11, 31, 41, 43*), markedly inhibited all platelet responses to α-thrombin, SFLLRN, and AYPGKF. These agents prevented ATP (and presumably ADP) secretion. They also nearly prevented PAR-1-(1−41) and PAR-4-(1−47) generation, prevented platelet aggregation, and eliminated the other platelet responses to the three agonists. Therefore, an agent that assures elevated intracellular [cAMP] inhibits generation of the platelet-derived PAR-cleaving proteinase *in situ* and the other platelet aggregation-related responses to the three PAR-dependent platelet agonists. As noted previously, agonist-mediated inhibition of platelet adenylate cyclase activity is one of the early and critical intracellular events seen during platelet activation (*11, 31, 42, 43, 52−54, 56, 57*). Further, the ability of forskolin and PGE1 to abrogate platelet responses to agonists by elevating [cAMP] is well established (*66, 67*). It is unclear why, in the presence of PGE_1, addition of AYPGKF to platelets results in more effective PAR-1-(1−41) and PAR-4-(1−47) release (than is observed when α-thrombin or SFLLRN is the agonist). It is also not clear why the more efficient generation of PAR-1-(1−41) and PAR-4-(1−47) in response to AYPGKF, in the presence of PGE_1 or forskolin, does not result in more responsive platelets. In the presence of PGE_1, AYPGKF-mediated generation of more PAR-1-(1−41) and PAR-4-(1−47) may be an example of a situation where generation of PAR-1-(1−41) and PAR-4-(1−47) cannot be equated with PAR-1 and -4 activation.

Inhibition of PKC with BIMI-1 markedly decreased PAR-1-(1−41) and PAR-4-(1−47) generation in response to α-thrombin, SFLLRN, or AYPGKF. It is unclear whether the ability of this PKC inhibitor to enhance α-thrombin- and AYPGKF-induced increases in intracellular $[Ca^{2+}]$ facilitated PAR-1-(1−41) and PAR-4-(1−47) generation. Nonetheless, PKC inhibition prevented ATP release and platelet aggrega-

tion in response to all three agonists. Therefore, PKC activation probably occurs before PAR-1-(1−41) and PAR-4-(1−47) generation, unmasking the PAR-1 and -4 tethered ligands, and the subsequent platelet responses can be effectively propagated. Since serine proteinases are normally synthesized as zymogens that are converted to active proteinases as required (*68*), we postulate that PKC may catalyze one or more protein phosphorylation reactions preceding activation of the proenzyme of the platelet proteinase that generates PAR-1-(1−41) and PAR-4-(1−47).

In contrast to PKC inhibition, PLC inhibition by U73122 minimally decreases PAR-1-(1−41) and PAR-4-(1−47) generation. Nonetheless, PLC inhibition effectively decreased the other platelet responses to α-thrombin or SFLLRN that occur after PAR-1-(1−41) and PAR-4-(1−47) are generated. Thus, while PLC is apparently not involved in PAR-1-(1−41) and PAR-4-(1−47) generation, this enzyme nonetheless helps to produce some of the other platelet aggregation-related responses to α-thrombin or SFLLRN. Specifically, platelet signaling reactions initiated principally via PAR-1, after PAR-1 is cleaved at $R^{41}S^{42}$, critically depend on PLC. Interestingly, PLC inhibition with U73122 did not decrease platelet aggregation initiated with AYPGKF. Thus, platelet aggregation-related reactions initiated principally via PAR-4, after this receptor is cleaved at $R^{47}G^{48}$, apparently are significantly less dependent on PLC than comparable PAR-1-dependent reactions. Inhibition of PI3-kinase with LY294002 did not decrease PAR-1-(1−41) and PAR-4-(1−47) generation in response to α-thrombin, SFLLRN, or AYPGKF. However, PI3-kinase inhibition significantly decreased ATP secretion initiated with α-thrombin or SFLLRN but not with AYPGKF. Furthermore, while PI3-kinase inhibition also decreased platelet aggregation to α-thrombin or SFLLRN, AYPGKF-induced aggregation was not affected. Thus, as was true for PLC, platelet signaling reactions initiated via PAR-4 do not require PI3-kinase, while initial PAR-1 activation-dependent signaling, at least in part, depends on the actions of PI3-kinase. Inhibition of PAR-1-dependent signaling by the PLC inhibitor, U73122, and the PI3-kinase inhibitor, LY294002, represents two other conditions under which normal generation of PAR-1-(1−41) and PAR-4-(1−47) via α-thrombin or SFLLRN does not necessarily coincide with PAR-1 and -4 activation.

We have provided evidence that a trypsin-like proteinase that is inhibitable by SBTI participates in PAR-1-(1−41) and PAR-4-(1−47) generation in response to α-thrombin, SFLLRN, or AYPGKF. Significantly, PAR-1-(1−41) and PAR-4-(1−47) generation in response to α-thrombin, SFLLRN, or AYPGKF is inhibited most effectively only when agonist-induced secretion of platelet ATP is eliminated by PGE_1, forskolin, or a PKC inhibitor. In the absence of ATP secretion, platelet aggregation in response to α-thrombin was inhibited to ≤15% of control. Thus, unidentified ATP-dependent phosphorylation reactions catalyzed by PKC probably help to generate the platelet-derived proteinase that propagates PAR-1-(1−41) and PAR-4-(1−47) generation and, ultimately, activation of the two platelet thrombin receptors. The requirement of ATP release for effective generation of PAR-1-(1−41) and PAR-4-(1−47) is similar to the requirement of ATP release by human monocytes prior to inflammasome-mediated cleavage and the subsequent secretion of IL-1B and IL-18 (*69*).

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