Protease-activated Receptors 1 and 4 Are Shut Off with Distinct Kinetics after Activation by Thrombin* 

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We report that PAR1 and PAR4 signal with distinct temporal profiles. In transfected fibroblasts, PAR4 triggered substantially more phosphoinositide hydrolysis per activated receptor than PAR1 and was shut off more slowly than PAR1. Shutoff and internalization of PAR1 depends upon phosphorylation of its carboxyl tail upon receptor activation. In contrast to PAR1, phosphorylation of PAR4 was undetectable, and activation-dependent internalization of PAR4 was much slower than that seen for PAR1. Mutation of potential phosphorylation sites in the carboxyl tail of PAR1 enhanced PAR1 signaling, whereas analogous mutations in PAR4 had no effect. Thus PAR4 signaling is shut off less rapidly than PAR1, probably due to differences in receptor phosphorylation. PAR1 and PAR4 also signaled with distinct temporal profiles in platelets. PAR1 triggered a rapid and transient increase in intracellular calcium, whereas PAR4 triggered a more prolonged response. Together, the tempo of these responses accounted for that triggered by thrombin. Thus differences in the rates at which PAR1 and PAR4 are shut off allow thrombin to trigger intracellular signaling with distinct temporal characteristics.

Thrombin, a serine protease generated at sites of vascular injury, is the most potent activator of platelets (1, 2). This action of thrombin is thought to be critical for hemostasis and thrombosis. Cellular responses to thrombin are mediated by a family of G protein-coupled protease-activated receptors (PARs) (3). Cleavage of the amino-terminal exodomain of a PAR unMASKS a tethered ligand that binds to the body of the receptor to trigger signaling. Synthetic peptides that mimic these tethered ligands are agonists for their respective PARs and have been used as pharmacological probes of PAR function in various cell types.

Three thrombin-activated receptors, PAR1, PAR3, and PAR4, have been identified (4–8). PAR1 and PAR4 appear to account for most if not all thrombin signaling in human platelets (9). Both receptors are expressed by platelets, and activation of either PAR1 or PAR4 with their cognate agonist peptides was sufficient to trigger platelet ATP secretion and aggregation. PAR1-blocking antibodies inhibited platelet secretion and aggregation at low but not high concentrations of thrombin. PAR4-blocking antibodies alone had little effect on platelet activation, but inhibition of both PAR1 and PAR4 with blocking antibodies markedly attenuated platelet activation even at high concentrations of thrombin. In heterologous expression systems, higher concentrations of thrombin were required for cleavage of PAR4 than PAR1. Taken together, these observations suggest that PAR1 is the primary mediator of platelet activation at low concentrations of thrombin and that, in the absence of PAR1 function, PAR4 can mediate platelet activation at high concentrations of thrombin. This model raises the question of why two thrombin receptors are present in human platelets. Are PAR1 and PAR4 simply redundant, do they interact, or do they serve distinct or only partially overlapping functions?

Because PARs are activated irreversibly by proteolytic cleavage, they have the potential to signal indefinitely. Previous studies have shown, however, that PAR1 signaling is rapidly shut off after activation by thrombin. Initial uncoupling and internalization depend on PAR1 phosphorylation, and receptor recycling and “resignaling” is prevented by delivery of internalized receptors to lysosomes (10–16). Here we report that PAR4 signaling is markedly more prolonged than PAR1 signaling in both heterologous expression systems and human platelets. PAR4 failed to undergo agonist-triggered phosphorylation and was internalized much more slowly than PAR1. Hence, PAR1 and PAR4 differ in the rate at which they are shut off after activation. Expression of these two distinct receptors may thus serve as a means of specifying the tempo with which thrombin activates intracellular signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—Human α-thrombin was from Enzyme Research Laboratories. The PAR1 agonist peptide SFLLRN and the PAR4 agonist peptide AYPGKF were synthesized as carboxyl-terminal amides and purified by high pressure liquid chromatography (17). M1 and M2 anti-FLAG monoclonal antibodies and hirudin were from Sigma.

Assay medium was Dulbecco’s modified Eagle’s medium (University of California Cell Culture Facility) supplemented with 1 mg/ml bovine serum albumin and 10 mM HEPES buffer, pH 7.0. Serum-free medium was Dulbecco’s modified Eagle’s medium supplemented with 1 mg/ml bovine serum albumin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone.

Phosphate-buffered saline (PBS) was from Mediatec and contained 0.1 mg/ml CaCl2, 0.2 mg/ml KCl, 0.2 mg/ml KH2PO4, 0.1 mg/ml MgCl2, 8 mg/ml NaCl, and 1.2 mg/ml Na2HPO4, pH 7.0.

Receptor Expression in Fibroblasts—cDNAs encoding human PAR1 and human PAR4 in the mammalian expression vector pBJ1 were utilized (9, 18). All receptors contained FLAG epitopes displayed at their amino termini (18). Rat1 cell lines stably transfected with these
cDNAs were generated and cultured, and COS7 cells were transiently transfected as described (19).

Phosphoinositide (PI) Hydrolysis Assays—Cells plated in 24-well tissue culture plates were labeled overnight in serum-free medium with [3H]inositol (Amersham Pharmacia Biotech) as described (20, 21). Cells were incubated at 37 °C for various periods (see figure legends) in serum-free medium containing, where indicated, thrombin, 1 unit/ml hirudin, and/or 20 mM lithium chloride to block metabolism of inositol phosphates. Cells were then lysed, and the total amount of [3H]inositol phosphates released was measured as described (20, 21).

Assays of Surface Receptor Levels, Receptor Cleavage, and Internalization—Cells plated in 24-well dishes were washed once in PBS and fixed in 2% paraformaldehyde, and anti-FLAG antibody binding was then measured as an index of surface receptor level as described previously (22). Briefly, cells were incubated with M1 anti-FLAG antibody (2.5 μg/ml) in assay medium for 1 h. Cells were washed and then incubated with goat anti-mouse secondary antibody coupled to horseradish peroxidase (Bio-Rad) for 30 min. The cells were washed three times and then incubated for approximately 10 min with a solution containing a chromogenic substrate for horseradish peroxidase, which is converted to a product that absorbs light at 405 nm (1-step 2.2'-azinedi(3-ethylbenzthiazoline sulfonate), Pierce). This solution was then removed from the cells, and its absorbance was measured. A405 varies linearly with the density of FLAG epitope on the cell surface over the range observed in these experiments (23). Cell surface receptor levels were determined in parallel assays of cells plated together with those used for each signaling experiment shown. Mean A405 (n = 3) values for each cell type are presented in the individual figure legends.

To measure receptor cleavage, cells were washed in PBS, incubated with thrombin in assay medium at 37 °C, washed twice in PBS, and then fixed. Anti-FLAG antibody binding to surface receptors was then assayed as above. Because the FLAG epitope is removed by thrombin cleavage, FLAG antibody binding provides a measure of uncleaved surface receptors and allows the percentage cleavage to be calculated by comparison with untreated cells (18).

To measure receptor internalization (22), cells were washed in PBS, incubated with assay medium containing PAR1 or PAR4 agonist peptide at 37 °C, and fixed, and then anti-FLAG antibody binding to surface receptors was assayed as above. Internalization was indicated by a decrease in surface receptor level in comparison with untreated cells.

Receptor Mutants—PAR1:S/T → A is a human PAR1 mutant in which all serine and threonine residues in the receptor’s cytoplasmic tail are replaced by alanines (10, 21). PAR4:S/T → A is the analogous human PAR4 mutant. A cDNA encoding hPAR4 in which serine residues 359, 366, 369, 374, 381, and 382, and threonine residues 363 and 379 were replaced by alanines was constructed by subcloning a synthetic insert generated by ligating three pairs of oligonucleotides to the PvuII site (5') and an XbaI site (3') in the vector.

Receptor Phosphorylation—Except where noted, receptor phosphorylation was assayed as described previously (11). Briefly, cells cultured in 6-well dishes were labeled with 250 μCi/well [32P]orthophosphate (NEN Life Science Products) at 37 °C for 3 h. Next, cells were stimulated with agonist peptides, washed in PBS, and lysed in an immunoprecipitation buffer (11) that included 1% Triton X-100, protease inhibitors, and phosphatase inhibitors. This and all subsequent steps were performed at 4 °C. For each sample analyzed, cells in two wells that had been treated identically were lysed in 0.75 ml of buffer/well and the lysates combined. The lysates were cleared by centrifugation and then 7.5 μg of M2 anti-FLAG antibody and 25 μl of protein G-Sepharose (Sigma) were added. These mixtures were incubated for 90 min and then washed three times. The beads were resuspended in 84 μl immunoprecipitation buffer and eluted with FLAG epitope peptide (sequence DYKDDDDK (Sigma), final concentration 0.2 mg/ml) for 30 min. Beads were then washed and then subjected to electrophoresis on 7.5% SDS-polyacrylamide gel. Proteins were then transferred to a nitrocellulose filter. The filter was incubated in a blocking solution of phosphoproteins and immunoblotted with anti-FLAG antibody to detect receptor protein. Bound antibody was detected using the ECL+ reagent (Amersham Pharmacia Biotech).

Platelet Studies—Washed human platelets were prepared from freshly drawn blood as described (9) and loaded with the calcium sensitive dye FURA-2 AM (Molecular Probes) as described (24, 25) and then washed again. To initiate PAR4 or PAR1 desensitization, platelets were treated for 30 min at room temperature with the PAR4 agonist peptide AYPGKF (500 μM) (17) or with the PAR1 agonist peptide SFLLRN (100 μM) (9). Platelets were then stimulated with thrombin or an agonist peptide, and intracellular calcium mobilization was measured by fluorometry (24, 25). To block platelet aggregation, 0.1 mM EGTA was added immediately prior to each calcium mobilization assay, and 0.1 mM CaCl2 was added immediately prior to cell lysis (24).

Statistics—Unless otherwise noted, each data point represents the mean ± S.D. (n = 3).

RESULTS AND DISCUSSION

We first compared PAR1- and PAR4-mediated signaling in Rat1 fibroblasts. This allowed signaling to be assayed in a system in which the levels of cell surface PAR1 and PAR4 could be accurately compared. To accomplish this, the cells were stably transfected with receptors displaying identical FLAG epitopes at their amino termini and the binding of an anti-FLAG monoclonal antibody to the cell surface was measured using an enzyme-linked secondary antibody.

We first examined thrombin-triggered PI hydrolysis in transfected Rat1 cells (Fig. 1). Maximal thrombin-triggered PI hydrolysis in cells expressing PAR4 was reliably higher than that seen in cells expressing PAR1, even when PAR4 was expressed at a lower density than PAR1 on the cell surface. For example, the cell line PAR4.1 reliably displayed 2.5-fold more PI hydrolysis than did a cell line expressing PAR1 despite expressing only 38% as much cell surface receptor. Similarly, a second PAR4-expressing cell line (PAR4.2) generated significantly more signal than the PAR1-expressing cells despite expressing only 18% as much surface receptor. Qualitatively similar results were obtained in transiently transfected COS7 cells (Fig. 4) and in a stably transfected lung fibroblast cell line derived from a PAR1 knockout mouse (17). Thus, on a per cell basis, PAR4 is more effective than PAR1 in triggering PI hydrolysis.
Each activated PAR4 molecule might yield more net PI hydrolysis than each PAR1 by either generating more signal per unit time while active and/or by each activated receptor signaling longer before being shut off. Accordingly, we also compared PAR1 and PAR4 shutoff (Fig. 1). Rat1 cell lines expressing PAR1 or PAR4 were incubated with thrombin for 1 h without lithium chloride. Under these conditions, receptors are cleaved and activated and PI hydrolysis is stimulated, but due to the absence of lithium, inositol phosphates fail to accumulate. After this initial incubation, thrombin was removed by washing, hirudin was added to inactivate any residual thrombin, and the cells were incubated for an additional hour in the presence of lithium. Inositol phosphates accumulated during this second incubation thus reflect ongoing signaling by receptors that had been activated by thrombin during the first incubation. Because the concentration of thrombin used is sufficient to cleave nearly all cell surface receptors within 10 min (not shown), the majority of receptors would have had substantial time to shut off. As described previously (11, 26, 27), PAR1 showed efficient shutoff in this assay. The signal generated when the thrombin and lithium incubations were sequential was only 20% of the signal generated when thrombin and lithium were presented simultaneously. In contrast, when PAR4-expressing cell lines were examined, the signal observed with sequential incubations was ~80% of that seen with thrombin and lithium together (Fig. 1). These data suggest that each activated PAR4 molecule signals for a longer time than PAR1.

To obtain a more detailed assessment of the tempo of PAR1- and PAR4-mediated PI hydrolysis, Rat1 cells expressing PAR1 or PAR4 were treated with thrombin for 10 min in the presence of lithium. Thrombin was then removed, hirudin was added, and the cells were further incubated in the presence of lithium. Accumulated [3H]inositol phosphates were measured after various periods. This allowed signaling triggered by receptors activated in the first 10 min to be monitored in the absence of additional receptor activation. Under these conditions, the rate of PI hydrolysis triggered by PAR4 decreased abruptly by approximately 7-fold upon removal of thrombin, consistent with previous observations (18). By contrast, the rate of PI hydrolysis triggered by PAR4 was virtually unchanged after removal of thrombin. In this experiment, PAR1 was expressed on the cell surface at a greater level than PAR4 and a greater fraction of PAR1 molecules were cleaved than PAR4 molecules (see Fig. 2 legend). Hence, the rate at which signaling in response to PAR1 activation is shut off is much greater than the rate at which PAR4 signaling is shut off.

Upon activation, many G protein-coupled receptors are rapidly phosphorylated and then bind arrestins. Arrestin binding serves to both prevent further interaction with G proteins and to recruit activated receptors to clathrin-coated pits for internalization (28–30). PAR1 is rapidly phosphorylated upon activation then internalized and sorted to lysosomes (10, 12–16), and PAR1 phosphorylation is important for both rapid shutoff of signaling and internalization (11, 19, 21, 26, 27). Given the slow shutoff of PAR4 signaling, these observations raise the question of whether PAR4 might show decreased phosphorylation and internalization compared with PAR1. To assay receptor phosphorylation, the cell lines described above were labeled with [32P]orthophosphate and stimulated with the PAR1 agonist SFLLRN or the PAR4 agonist AYPGKF (Fig. 3). AYPGKF, a derivative of the PAR4-tethered ligand GYPGQV, triggers PAR4 signaling in fibroblasts to the same extent as thrombin, whereas GYPGQV is a weaker agonist (17). Both receptors were immunoprecipitated from cell lysates via the FLAG epitope tag on their amino termini, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography and FLAG immunoblot. Because PAR1 and PAR4 were detected using the same antibody to the same epitope at the same relative position in the receptor protein, the relative intensities of the PAR1 and PAR4 bands on the immunoblot likely reflect the relative amounts of PAR1 and PAR4 protein recovered in the immunoprecipitates. PAR1 displayed robust agonist-triggered phosphorylation (Fig. 3, upper panel, lanes 4 and 5). Anti-FLAG immunoblot readily detected PAR1 at the same molecular weight as the phosphorylated band in immunoprecipitates from PAR1-transfected but not untransfected cells (Fig. 3, lower panel, lanes 4 and 5). PAR4 protein was also apparent by immunoblot of PAR4-transfected but not untransfected cells. The amount of PAR4 recovered in immunoprecipitates from lysates of PAR4-expressing cells was less than the amount of PAR1 recovered from PAR1-expressing cells, consistent with the relatively lower expression level of PAR4 in the cell lines used. To compensate for this difference, the amount of PAR1 immunoprecipitate analyzed was reduced by a factor of 10 (Fig. 3, lanes 1 and 2). Under these conditions, the amount of PAR1
detected by immunoblot was less than the amount of PAR4 (compare lanes 1 and 2 with lanes 6 and 7), but agonist-triggered phosphorylation of PAR1 was still clearly apparent (lane 2). These data indicate that PAR4 phosphorylation would have been detected if PAR4 were phosphorylated to an extent comparable to PAR1. We conclude that PAR4 is either not phosphorylated or is phosphorylated at a rate or to an extent markedly less than PAR1.

We also assessed the importance of potential phosphorylation sites in PAR4 by mutagenesis. A PAR1 mutant (PAR1:S/T → A), in which the 13 serines and threonines in its cytoplasmic tail were replaced with alamines, was not phosphorylated, triggered substantially more PI hydrolysis upon activation than wild-type PAR1, and exhibited a delay in receptor shutoff (10, 11, 21). The cognate PAR4 mutant (PAR4:S/T → A), in which the 8 serines and threonines in its cytoplasmic tail were replaced with alamines, was generated to determine if a similar change in signaling properties would result. COS7 cells expressing wild-type PAR4 or PAR4:S/T → A showed similar increases in PI hydrolysis when stimulated with 30 nM thrombin (Fig. 4) or with submaximal concentrations of thrombin (not shown). By contrast, PAR1:S/T → A mediated substantially more PI hydrolysis than did wild-type PAR1 (Fig. 4). These data suggest that PAR4 is either not phosphorylated on its cytoplasmic tail or such phosphorylation does not significantly attenuate its signaling.

Agonist-triggered internalization of PAR1 is dependent on its phosphorylation, as inferred from the observation that PAR1:S/T → A was defective in this function (19). If PAR4 phosphorylation is less robust than PAR1 phosphorylation, one might anticipate that agonist-triggered internalization of PAR4 would be similarly decreased. To test this prediction, receptor internalization was assayed in Rat1 cells (Fig. 5). A ∼50% decrease in cell surface PAR1 was observed within 20 min of stimulation with SFLLRN and a ∼80% decrease was observed after 1 h. By contrast, only a ∼35% decrease in cell surface PAR4 was observed even 1 h after stimulation with AYPGKF. Thus PAR4 undergoes less robust agonist-triggered internalization than PAR1, consistent with PAR4’s slower shutoff and lack of phosphorylation.

To determine if the different tempos of signaling exhibited by the PAR1 and PAR4 in heterologous expression systems might be seen in cells in which both receptors are endogenously expressed, PAR1- and PAR4-mediated calcium mobilization was examined in human platelets (Fig. 6). Thrombin triggered a rapid increase in the intracellular calcium concentration followed by a relatively slow return to baseline. The t½ for this

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**Fig. 3.** Phosphorylation of PAR1 and PAR4. [32P]Orthophosphate-labeled cells were stimulated for 5 min at 37 °C with appropriate agonist peptides or left unstimulated. Immunoprecipitates of cell lysates were eluted as described under “Experimental Procedures.” The indicated volume of eluate from each immunoprecipitate was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (top panel) and immunoblot (bottom panel). Molecular masses (in kilodaltons) are shown at the left. Lane 1, 5 μl of eluate, untransfected PAR1-transfected cells. Lane 2, 5 μl of eluate, PAR1-transfected cells stimulated with SFLLRN (100 μM). Lane 3, 5 μl of eluate, untransfected Rat1 cells. Lane 4, 5, and 8, 50 μl of the samples analyzed in lanes 1, 2, and 3, respectively. Lane 6, 50 μl of eluate, untransfected PAR4-transfected cells. Lane 7, 50 μl of eluate, PAR4-transfected cells stimulated with AYPGKF (500 μM). Note that both PAR1 (empty arrow) and PAR4 (solid arrow) run at higher than their predicted molecular masses, presumably due to glycosylation and/or oligomerization. The intensities of the PAR1 and PAR4 bands on immunoblot were markedly reduced when intact cells were exposed to thrombin prior to lysis. Thrombin cleaves the FLAG epitope from the receptor, thus the bands shown likely result from receptors that resided on the cell surface. This experiment was replicated twice.

**Fig. 4.** Signaling by PAR1 and PAR4 phosphorylation site mutants. COS7 cells were transiently transfected with wild-type PAR1, wild-type PAR4, a PAR1 mutant in which all the serine and threonines in the cytoplasmic tail were replaced with alamines (PAR1:S/T → A), with an analogous PAR4 mutant (PAR4:S/T → A), or with empty pBJ expression vector (none). [3H]Inositol-labeled cells were incubated in serum-free medium containing either 500 μM AYPGKF (squares) or 100 μM SFLLRN (circles) at 37 °C for 15 min in the presence (filled bars) or absence (empty bars) of 30 nM thrombin. Cells were then washed twice in PBS and incubated for an additional 75 min in serum-free medium with 20 mM LiCl and 1 unit/ml hirudin. Accumulated [3H]inositol phosphates were then measured (mean ± S.D.; n = 4). The surface receptor levels (Acell) were 0.75, 0.77, 1.2, 1.2, and 0.01 for wild-type and mutant PAR1, wild-type and mutant PAR4, and empty vector-transfected cells, respectively. PAR4 and PAR4:S/T → A did display higher basal activity than did PAR1 in these cells; the basis for this difference is unknown. This experiment was replicated twice.

**Fig. 5.** PAR1 and PAR4 internalization. Rat1 cells lines expressing PAR4 (squares) or PAR1 (circles) were incubated in assay medium containing either 500 μM AYPGKF (squares) or 100 μM SFLLRN (circles) at 37 °C for the indicated times. The cells were then fixed, and the cell surface receptor levels were determined. For each cell line, the receptor level at each time point was expressed as a percentage of the level at time zero. The decrease in surface receptor level is thus an index of agonist-triggered receptor internalization. The actual surface receptor levels (Acell) were 1.4, 0.4, and 0.01 for PAR1, PAR4, and untransfected Rat1 cells, respectively. This experiment was replicated three times.
response was 253 s ($t_{1/2}$ is defined as the length of time between the addition of agonist and the time at which the intracellular calcium level returned halfway to baseline after reaching a peak level). PAR1 activation recapitulated the rapid rise in intracellular calcium triggered by thrombin but not the prolonged nature of the thrombin response. The PAR1 agonist SFLLRN triggered a rapid increase in intracellular calcium and a rapid return to baseline ($t_{1/2} = 54$ s). By contrast, PAR4 activation mimicked the prolonged rise in intracellular calcium triggered by thrombin but not the rapid rise. The PAR4 agonist AYPGKF triggered a relatively gradual increase in intracellular calcium that was followed by a gradual return to the baseline level ($t_{1/2} = 203$ s).

Calcium mobilization in response to thrombin was also measured in platelets in which either PAR1 or PAR4 was desensitized by prolonged incubation with the appropriate agonist peptide. Under these conditions, the contribution of the desensitized receptor to calcium mobilization is minimized (9, 17). The peak increases in intracellular calcium were reduced under these conditions, presumably due to prior calcium signaling during desensitization; however, the data again show differences in the tempo of PAR1 and PAR4 signaling (Fig. 6, d and e). When PAR4-desensitized platelets were stimulated with thrombin, relatively transient calcium mobilization was observed ($t_{1/2} = 66$ s), similar to that seen in naïve platelets activated with SFLLRN. When PAR1-desensitized platelets were stimulated with thrombin, the calcium level rose and decayed relatively slowly ($t_{1/2} = 390$ s), similar to that seen in naïve platelets activated with AYPGKF. These results suggest that PAR1 and PAR4 signal with different tempos in human platelets when activated by either thrombin or their agonist peptides. PAR1 triggers a rapid increase in cytoplasmic calcium followed by a rapid decay, whereas PAR4 triggers a more gradual and prolonged increase in cytoplasmic calcium. Thrombin-triggered calcium signaling in human platelets seems to be a composite of PAR1 and PAR4 signaling, with the rapid rise presumably being PAR1-mediated and the prolonged elevation being PAR4-mediated.

Curiously, measurement of PAR1- and PAR4-mediated calcium mobilization in transfected fibroblasts did not reveal differences in the rate at which elevations in intracellular calcium concentration returned to baseline (not shown). Specifically, cytoplasmic calcium levels returned very rapidly to baseline after an increase was triggered by activation of either receptor. Because striking differences in the rate at which PAR1- and PAR4-mediated PI hydrolysis was shut off were seen in the same cell lines, we conclude that the machinery for calcium homeostasis in fibroblasts allows only transient increases in cytoplasmic calcium and that, after a time, ongoing signaling at the level of PI hydrolysis is not reflected by sustained increases in cytoplasmic calcium in these cells. The molecular basis for the different tempos of calcium signaling seen in platelets and fibroblasts is unknown.

In summary, we report that PAR1 and PAR4 signal with distinct tempos in two systems. In transfected fibroblasts, PAR4 triggered more PI hydrolysis per cleaved and activated receptor and more ongoing PI hydrolysis after removal of thrombin than did PAR1. In human platelets, PAR4 activation triggered a more persistent elevation in cytoplasmic calcium than did PAR1. These observations suggest that PAR4 is uncoupled from G protein signaling more slowly than PAR1. In accord with this notion, PAR4 was either not phosphorylated upon activation or phosphorylated to a substantially lesser extent than PAR1, and PAR4 internalized much less robustly than PAR1. Mutation of potential phosphorylation sites in PAR4 had little effect on its signaling, whereas such mutations enhanced PAR1 signaling (Fig. 4) and attenuated both PAR1 and PAR4 signal with distinct tempos in several cell systems, per-
haps due to differences in the rate and/or extent of agonist-depen-
dent receptor phosphorylation.

The molecular basis for the lack of PAR4 phosphorylation is not known. PAR4 does have potential serine and threonine phosphorylation sites in its carboxyl tail, but it is possible that other features of the sequence or perhaps the structure of this region make PAR4 an intrinsically poor substrate for receptor kinases compared with PAR1. Alternatively, it is possible that the membrane microenvironment in which PAR4 resides or the βγ subunits and signaling molecules with which PAR4 interacts may dictate less efficient interactions with receptor kinases.

What is the biological significance of the differences in the kinetics of PAR1 and PAR4 signaling? Together, these receptors appear to account for the tempo of calcium signaling in platelets activated by high concentrations of thrombin, thus their signaling phenotypes appear to account for an important second messenger response in “real” cells. But why use two receptors with distinct tempos of signaling? The thrombin concentration required for PAR1 activation is lower than that for PAR4 (7–9). It may be advantageous for platelets that “see” only low concentrations of thrombin and experience subthreshold or partial activation via PAR1, as might occur in the bloodstream passing by an incipient thrombus, to become quiescent again by virtue of rapid shutoff of PAR1 signaling. By contrast, platelets exposed to sufficient thrombin to activate PAR4 may have a very high probability of being incorporated into a thrombus, making shutoff unattainable. However, high thrombin concentrations are sufficient to trigger robust platelet aggregation and via PAR1 alone (9). When then, does PAR4 contribute? It is possible that prolonged signaling mediated by PAR4 is important for platelet responses other than secretion and aggregation such as compaction of a loose platelet aggregate into a tight mass and/or fibrin clot retraction. Alternatively, PAR4 may provide a means for platelets to respond to proteases other than thrombin such as cathepsin G (31). In this regard, the poor shutoff of PAR4 may represent an adaptation that allows robust signaling even when relatively few receptors are activated by thrombin or another protease.

The differences in the kinetics of PAR1 and PAR4 signaling may also relate to differences in the tissue distribution of the two receptors. Thus far, PAR4 is known to function only in platelets, but Northern analysis suggests that it is expressed in other tissues (8). Perhaps PAR4 is expressed only in cell types in which thrombin triggers a sustained change in cellular structure or function. PAR1, however, is expressed in endothelial cells (32–34), which presumably sense and respond to thrombin repeatedly over time and thus require rapid termination of signaling when thrombin is removed from their environment. Finally, the differences in the kinetics of PAR1 and PAR4 signaling may allow thrombin to trigger distinct events, such as specific transcriptional programs, depending on which receptor is activated (35). Thus the existence of two receptors that signal with distinct kinetics might allow thrombin to elicit distinct responses in cell types that express different receptors or even produce distinct effects in the same cell type depending on thrombin concentration.

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REFERENCES

1. Davey, M., and Luscher, E. (1967) Nature 216, 857–858
2. Berndt, M., and Phillips, D. (1981) in Platelets in Biology and Pathology (L. F. Brass, ed.) pp. 43–74, Elsevier Biomedical Press, Amsterdam
3. Coughlin, S. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11023–11027
4. Vu, T.-K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 107–1086

5. Rasmussen, U. B., Vouret-Craviari, V., Jallat, S., Schlesinger, Y., Pagers, G., Pavirani, A., Lecocq, J. P., Pouyssegur, J., and Van Obbergen-Schilling, E. (1991) FEBS Letts. 288, 123–128
6. Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T., and Coughlin, S. R. (1997) Nature 386, 502–506
7. Kahn, M. L., Zheng, Y. W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, R. V., Jur, T. M., Tan, C., and Coughlin, S. R. (1996) Nature 384, 686–684
8. Xu, W. P., Andersen, H., Whitmore, T. E., Presnell, S. R., Yee, D. P., Ching, A., Gilbert, T., Davie, E. W., and Foster, D. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6642–6646
9. Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H., and Coughlin, S. R. (1999) J. Clin. Invest. 103, 879–887
10. Ishii, K., Chen, J., Ishii, M., Koch, W. J., Freedman, N. J., Lefkowitz, R. J., and Coughlin, S. R. (1992) J. Biol. Chem. 267, 1125–1130
11. Hammes, S. R., Shapiro, M. J., and Coughlin, S. R. (1999) Biochemistry 38, 2486–2493
12. Vouret-Craviari, V., Grall, D., Chambard, J. C., Rasmussen, U. B., Pouyssegur, J., and Van Obbergen-Schilling, E. (1995) J. Biol. Chem. 270, 8367–8372
13. Hein, L., Ishii, K., Coughlin, S. R., and Kobilka, B. K. (1994) J. Biol. Chem. 269, 27719–27726
14. Hoxie, J. A., Abuja, M., Belmonte, E., Pizarro, S., Parton, R., and Brass, L. F. (1993) J. Biol. Chem. 268, 13756–13763
15. Tao, J., and Coughlin, S. R. (1993) J. Biol. Chem. 274, 2216–2224
16. Brass, L. F., Pizarro, S., Abuja, M., Belmonte, E., Bianchard, N., Stadel, J. M., and Hoxie, J. A. (1994) J. Biol. Chem. 269, 2943–2952
17. Fox, B. R., Weiss, E. J., Shapiro, M. J., Hung, W., and Coughlin, S. R. (2000) J. Biol. Chem. 275, 19728–19734
18. Ishii, K., Hein, L., Kobilka, B., and Coughlin, S. R. (1993) J. Biol. Chem. 268, 9780–9786
19. Shapiro, M. J., Trejo, J., Zeng, D., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 32874–32880
20. Hung, D. T., Vu, T.-K. H., Nelenk, N. A., and Coughlin, S. R. (1992) J. Cell Biol. 116, 827–832
21. Nanevica, T., Wang, L., Chen, M., Ishii, M., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 702–706
22. Shapiro, M. J., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 29009–29014
23. Ishii, K., Gerstzen, R., Zheng, Y. W., Welch, J. B., Turck, C. W., and Coughlin, S. R. (1995) J. Biol. Chem. 270, 16435–16440
24. Sage, S. O. (1996) Use of Fluorescent Indicators to Measure Intracellular Ca2+ and Other Ions in Platelets, A Practical Approach, pp 67–90, IRL Press, Oxford
25. Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T., and Coughlin, S. R. (1997) Nature 386, 502–506
26. Ishihara, H., Luscher, E. (1967) Nature 215, 161–165
27. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) Life Sci. 62, 1561–1665
28. Sambrano, G. R., Huang, W., Faruqui, T., Mahrus, S., Craik, C., and Coughlin, S. R. (2000) J. Biol. Chem. 275, 6819–6823
29. Ngaija, J. R., and Jaffe, E. A. (1991) Biochem. Biophys. Res. Comm. 179, 1656–1661
30. Rasmussen, U. B., Vouret-Craviari, V., Auburger, P., Pouyssegur, J., and Van Obbergen-Schilling, E. (1995) J. Biol. Chem. 270, 4813–4821
31. Trejo, J., Hammes, S. R., and Coughlin, S. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13698–13703
32. Krupnick, J. G., and Benovic, J. L. (1998) Ann. Rev. Pharmacol. Toxicol. 38, 289–319
33. Letkowitz, R. J. (1998) J. Biol. Chem. 273, 18677–18680
34. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) Life Sci. 62, 1561–1665
35. Coughlin, S. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11023–11027
36. Woolkalis, M. J., DeMelfi, T. J., Blanchard, N., Hoxie, J. A., and Brass, L. F. (1995) J. Biol. Chem. 270, 9868–9875
37. Chih, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shelton, J. M., Wu, H., Zhu, W., Bassel-Duby, R., and Williams, R. S. (1998) Genes Dev. 12, 2499–2509