Protease-activating Receptor-4 Induces Full Platelet Spreading on a Fibrinogen Matrix

**INVOLVEMENT OF ERK2 AND p38 AND Ca^{2+} MOBILIZATION**

Received for publication, October 20, 2006; and in revised form, December 21, 2006. Published, JBC Papers in Press, January 2, 2007, DOI 10.1074/jbc.M609881200

Alexandra Mazharian, Séverine Roger, Eliane Berrou, Frédéric Adam, Alexandre Kauskot, Paquita Nurden, Martine Jandrot-Perrus, and Marijke Bryckaert

From 1 U689 INSERM, IFR139, Hôpital Lariboisière, 8 rue Guy Patin, 75010 Paris, France, 2 U698 INSERM, Hôpital Bichat, 75018 Paris, France, and 3 IFR4, Laboratoire d’Hématologie, Hôpital Cardiologique, 33604 Pessac, France

Although the involvement of protease-activating receptor PAR1 and PAR4 is well established in platelet aggregation, their role in platelet adhesion and spreading has yet to be characterized. We investigated platelet adhesion and spreading on a fibrinogen matrix after PAR1 and PAR4 stimulation in correlation with the activation of two MAPKs, ERK2 and p38. Of the two PAR-activating peptides (PAR-APs), PAR1-AP and PAR4-AP, which both induce adhesion, only PAR4-AP induced full platelet spreading. Although both PAR1-AP and PAR4-AP induced ADP secretion, which is required for platelet spreading, only PAR4-AP induced sustained Ca^{2+} mobilization. In these conditions of PAR4 induction, ERK2 and p38 activation were involved in platelet spreading but not in platelet adhesion. p38 phosphorylation was dependent on ADP signaling through P2Y12, its receptor. ERK2 phosphorylation was triggered through integrin αIIbβ3 outside-in signaling and was dependent on the Rh pathway. ERK2 and p38 activation induced phosphorylation of the myosin light chain and actin polymerization, respectively, necessary for cytoskeleton reorganization. These findings provide the first evidence that thrombin requires PAR4 for the full spreading response. ERK2 and p38 and sustained Ca^{2+} mobilization, involved in PAR4-induced platelet spreading, contribute to the stabilization of platelet thrombi at sites of high thrombin production.

Platelet activation plays a crucial role in hemostasis and in the pathogenesis of thrombosis. Thrombin is one of the most potent agonists for platelet activation, mediating cellular effects via glycoprotein Ibα and protease-activating receptors (PARs) coupled to G proteins (1–4). Two PARs, PAR1 and PAR4, are present in human platelets (5–8). PAR1, a high affinity receptor, mediates platelet activation at low concentrations of thrombin, whereas PAR4, a low affinity receptor, is involved in thrombin signaling at high concentrations.

After thrombin binding, PAR1 is cleaved at a specific site located in its N-terminal exodomain. The cleaved N terminus is a tethered peptide ligand that activates PAR1 (5). The synthetic peptide SFLLRN-NH₂ (PAR1-AP), which corresponds to the first 6 residues of the new N-terminal sequence of PAR1, and AYPGKF-NH₂ (PAR4-AP), the equivalent for PAR4, can also activate their cognate receptors without the need for receptor cleavage by the protease (8, 9). Stimulation of either PAR1 or PAR4 with these peptide ligands triggers most of the platelet responses elicited by thrombin (8–10). However, unlike thrombin, PAR1-AP is not a full agonist for platelet activation and requires secreted ADP for a complete platelet response, whereas PAR4 is activated without requiring ADP to be effective (11). PAR1 has been shown to induce a rapid transient spike in Ca^{2+} mobilization, whereas PAR4 induces a robust prolonged response (12). These differences in the timing and magnitude of the two PAR signals suggest distinct roles. Both contribute to thrombin-induced platelet activation, but they contribute, either early or late, to platelet activation. Although the receptors have been identified, the molecular events leading to PAR-AP-induced platelet aggregation are not yet fully characterized, and the signaling events triggered by the activating peptides remain unclear.

It is well established that thrombin induces MAPK activation in human platelets (13, 14). Human platelets contain several members of the MAPK family, including ERK2, p38, and JNK1, 2, 3 by von Willebrand factor binding to its receptor, glycoprotein Ib-IX-V, is dependent on protein kinase G and MAPK. With other agonists such as low doses of collagen (23) and thrombin (24), ERK2 plays a crucial role in platelet aggregation. The platelet P2X1 ion channel-dependent activation of ERK2 contributes to collagen-induced...
platelet activation by enhancing platelet secretion; it does this by enhancing early myosin light chain kinase phosphorylation (25). In transgenic mice overexpressing human P2X1, pre-injection of an inhibitor of ERK2 activation fully protects against thrombosis (26). The other MAPK, p38, plays a role in collagen-induced platelet aggregation (27), von Willebrand factor-induced platelet activation (28), and procoagulant activity (29). p38 is also involved in platelet activation induced by the thromboxane A2 analog or by 8-isoprostaglandin F2α, a marker of oxidative stress (30).

In this study, we examined the role of ERK2 and p38 induced by PAR1 and PAR4 agonists in platelet adhesion and spreading on a fibrinogen matrix. We show that PAR4-AP (but not PAR1-AP) induces full platelet spreading on a fibrinogen matrix. Only PAR4-AP induces sustained Ca2+ mobilization required for spreading. Moreover, the MAPKs ERK2 and p38, necessary for platelet spreading, are linked to the signaling pathway induced by the secretion of ADP. Activation of p38, required for polymerization of actin, is dependent on the signaling pathway induced by ADP with its own P2Y12 receptor. ERK2, required for myosin light chain phosphorylation induced by PAR4-AP, is dependent on integrin αIIbβ3 outside-in signaling. Both MAPKs act on cytoskeleton rearrangement required for platelet spreading.

### EXPERIMENTAL PROCEDURES

**Materials**—The thrombin receptor-derived peptides SFLLRN-NH2 (H-Ser-Phe-Leu-Leu-Arg-Asn-NH2: PAR1-AP) and AYPGKF-NH2 (H-Ala-Tyr-Pro-Gly-Lys-Phe-NH2: PAR4-AP) were purchased from Bachem (Weil am Rhein, Germany). Leupentin, aprotinin, Me2SO, apyrase (grade VII), MRs 2179, Y-27632, BAPTA-1/AM, and fluorescein isothiocyanate-phalloidin were purchased from Sigma. Fluorescein isothiocyanate-labeled PAC1, a ligand-mimetic integrin αIIbβ3-specific monoclonal antibody that binds specifically to activated αIIbβ3, was purchased from BD Biosciences. AR-C69931MX was generously provided by Dr. B. Humphries (AstraZeneca).

Abciximab was obtained from Lilly (Suresnes, France). Human α-thrombin was purified as reported previously (31). Fibrinogen was obtained from HYPHEN BioMed SAS (Andresy, France). Indomethacin was obtained from Cayman Chemical (Ann Arbor, MI). A specific inhibitor of human p38 (SB203580) was obtained from Calbiochem-Novabiochem. The MEK inhibitor U0126 and the polyclonal antibody directed against the phosphorylated form of p38 were purchased from Promega Corp. (Madison, WI). Alexa Fluor 488-labeled phalloidin and Oregon Green 488 BAPTA-1/AM was obtained from Molecular Probes (Eugene, OR). The monoclonal antibody directed against tubulin was obtained from Sigma. The polyclonal antibodies directed against the phosphorylated forms of ERK and myosin light chain (MLC) were purchased from BIOSOURCE (Camarillo, CA). The anti-phospho-Hsp27 polyclonal antibody was obtained from Stressgen Bioreagents (Victoria, British Columbia, Canada). Peroxidase-conjugated AffiniPure donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**Isolation of Platelets**—Venous blood was collected from healthy donors, who were free of medication for at least 2 weeks prior to blood collection. Platelet-rich plasma was obtained by centrifugation of whole blood at 120 × g for 15 min at 20 °C, and platelets were isolated as described previously (23). The platelet pellet was resuspended in 10 mM HEPES (pH 7.4), 140 mM NaCl, 3 mM KCl, 5 mM NaHCO3, 0.5 mM MgCl2, and 10 mM glucose.

**Immunoblotting**—Samples were immunoblotted as described previously (23). Briefly, platelets were lysed in SDS denaturing buffer (100 mM NaCl, 50 mM Tris, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 100 μM phenylarsine oxide, 1% SDS, 5 μg/ml leupentin, and 10 μg/ml aprotinin (pH 7.4)). Proteins were subjected to SDS-PAGE on acrylamide gels and transferred to nitrocellulose. The filters were incubated with various primary antibodies: anti-phospho-ERK (1:5000), anti-phospho-p38 (1:8000), anti-phospho-MLC (1:1000), anti-phospho-Hsp27 (1:3000), and anti-tubulin (1:4000). Immunoreactive bands were visualized with enhanced chemiluminescence detection reagents (Pierce).

**Static Platelet Deposition**—A platelet suspension (106 platelets/ml) containing indomethacin (5 μM) was preincubated for 15 min at 37 °C with U0126 (10 μM), SB203580 (10 μM), or Me2SO. The platelets were then stimulated or not with either PAR1-AP (2–100 μM) or PAR4-AP (2–100 μM) and immediately plated (50 μl) by incubation for 1 h at 20 °C in wells precoated with fibrinogen (200 μg/ml). Nonspecific adhesion was determined in wells precoated with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline. Platelet deposition was quantified by an acid phosphatase assay as described previously (30, 32).

**Platelet Spreading**—A platelet suspension (5 × 106 platelets/ml) containing indomethacin (5 μM) was preincubated for 15 min at 37 °C with U0126 (10 μM), SB203580 (10 μM), or Me2SO. The platelets were then stimulated or not with either PAR1-AP (50 μM), PAR4-AP (100 μM), or human α-thrombin (0.3 or 25 nM) and immediately plated in wells precoated with fibrinogen (200 μg/ml). The platelets were incubated for 1 h at 20 °C. Unbound platelets were removed, and then adherent platelets in the absence of aggregates were fixed with 4% paraformaldehyde, permeabilized, and stained by incubation for 30 min with Alexa Fluor 488-labeled phalloidin. The platelets were visualized under an epifluorescence microscope (Nikon Eclipse 600), and the cell surfaces were analyzed using NIH Image software (Version 1.67b; rsb.info.nih.gov/nih-image/). Data are expressed as a percentage of the cell area determined in the absence of PAR-AP.

**Platelet Secretion**—Platelet-rich plasma was incubated with 14C-labeled 5-hydroxytryptamine (5-HT; 0.5 μCi/10 ml) for 30 min at 37 °C. Platelets were then isolated as described below. After adhesion, the supernatants were centrifuged for 10 min and removed for liquid scintillation counting.

**Platelet Ca2+ Measurement**—Washed platelets (4 × 109/ml) were loaded with the Ca2+-sensitive dye Oregon Green 488 BAPTA1-AM (10 μM) for 45 min at 37 °C. Loaded platelets were washed, resuspended at 1 × 109/ml, and left at room temperature for 30 min before experimentation. Platelets were allowed to sediment onto fibrinogen for 30 min. Suspended platelets were then eliminated. Fluorescence changes in Ca2+ were measured after the addition of PAR1 and PAR4 peptides.
Protease-activating Receptor PAR4 and MAPKs
to adherent platelets. Changes in calcium concentration wereanalyzed by confocal microscopy using a Zeiss LSM 510inverted confocal microscope with an LD Achromplan ×63objective (numeric aperture, 0.8). Oregon Green BAPTA-1/AMwas excited at the 494-nm wavelength of an argon laser,and fluorescence was measured at 523 nm. Zeiss confocalsoftware running on Windows NT was used to controlled thescanner module and to perform image analysis. Images wereacquired every 2 s.

Flow Cytometry—A platelet suspension (100 μl, 10⁸ platelets/ml) was preincubated for 15 min at 37 °C with U0126 (10 μM),SB203580 (10 μM), or Me₂SO. The platelets were then stimulated or not with PAR4-AP (100 μM) in the presence of fluorescein isothiocyanate-labeled PAC1 (20 μg/ml) for 30 min atroom temperature. The reaction was stopped by the addition of1% paraformaldehyde and was analyzed using a FACSCaliburflow cytometer (BD Biosciences).

Determination of F-actin Content—A platelet suspension(10⁸ platelets/ml) containing indomethacin (5 μM) was preincubated for 15 min at 37 °C with U0126 (10 μM), SB203580 (10 μM), or Me₂SO. The platelets were then stimulated or not with PAR4-AP (100 μM) and plated (50 μl) by incubation for 1 h at20 °C in wells precoated with fibrinogen (200 μg/ml). Platelets were fixed with 4% paraformaldehyde, permeabilized, andincubated for 30 min at room temperature with 2 μM TRITC-phalloidin to detect F-actin. The platelets were then washedfive times. Bound TRITC-phalloidin was quantified using aFluoroscan Ascent FL fluorescence spectrophotometer (Lab-systems) with excitation at 544 nm and emission at 590 nm.

Statistics—Results are expressed as the means ± S.E. of atleast three independent experiments. Statistical significancewas assessed with Student’s t test for paired comparisons.

RESULTS
PAR4 Is Required for Full Platelet Spreading on a FibrinogenMatrix—Thrombin is a potent agonist of platelet activation. Inthis study, we compared the ability of the thrombin-activatedreceptors PAR1 and PAR4 to trigger platelet adhesion andspreading on a fibrinogen matrix. Platelets stimulated or not with various doses of PAR-APs (PAR1-AP and PAR4-AP) wereimmediately deposited in wells precoated with fibrinogen (200 μg/ml) for 1 h. At a platelet concentration at which platelet aggregates were absent (10⁶/ml), PAR1-AP and PAR4-APinduced a similar increase in adhesion (173 and 192% of thecontrol, respectively; 15.3 and 16.9 × 10⁵ platelets versa. 8.8 × 10⁵ platelets for the control unstimulated platelets) (Fig. 1) irrespectiv eof peptide concentration (1–100 μM).

To test the ability of PAR-APs to induce platelet spreading,very low concentrations of platelets (5 × 10⁵/ml) werestimulated with PAR1-AP (50 μM) and PAR4-AP (100 μM) andimmediately deposited in wells precoated with fibrinogen.Under these conditions, only isolated platelets were observed.A small increase in platelet spreading (120% of the control) withPAR1-AP was observed. By contrast, PAR4-AP increased platelet spreading by 163% compared with the unstimulated control(Fig. 2A). Similar spreading was obtained when platelets wereallowed to sediment on fibrinogen for 30 min, followed by theaddition of PAR-APs to adherent platelets (see Fig. 5A). To
directly demonstrate that platelet spreading coupled to thrombin was mostly dependent on PAR4, platelets were pretreated or not for 30 min at 37 °C with an excess of PAR1-AP (100 μM) and then stimulated with human thrombin at 0.3 and 25 nM, involving PAR1 and PAR1/PAR4, respectively (34). Thrombin (0.3 nM) and PAR1-AP (50 μM) showed weak but comparable spreading (116 ± 3% for thrombin versus 117 ± 2% for the PAR1 peptide) (Fig. 2B). After PAR1 desensitization, platelet spreading induced by thrombin (0.3 nM) returned to the baseline level (102 ± 2%). At a higher thrombin concentration (25 nM), thrombin and PAR4-AP (100 μM) induced similar spreading (150 ± 4% for thrombin versus 148 ± 3% for PAR4-AP), which was not modified with PAR1-desensitized platelets (147 ± 4% for thrombin and 145 ± 3% for PAR4-AP). These results show that PAR4 is more potent in enhancing platelet spreading than is PAR1.

PAR1-AP and PAR4-AP Induce Comparative Secretion—To explore the difference observed between PAR1-AP- and PAR4-AP-induced platelet spreading, we examined secretion, which has been described as a source of secondary messenger after PAR4-AP-induced platelet spreading (120%). PAR1-AP (2–100 μM) or PAR4-AP (2–100 μM) and immediately plated for 1 h in wells precoated with fibrinogen (200 μg/ml). The “0” PAR-AP point corresponds to control unactivated platelets. Data are expressed as the means ± S.E. of at least three independent experiments.
spreading by 90 and 66%, respectively, compared with the control in the absence of peptide, whereas SB203580 inhibited platelet spreading induced by PAR1-AP and PAR4-AP by 70 and 58%, respectively. We checked that the phosphorylation levels of ERK2 and Hsp27, an indirect substrate of p38, were inhibited in the presence of U0126 and SB203580, respectively. In parallel, the phosphorylation levels of p38 and ERK2 were examined with and without PAR1-AP (50 μM) and PAR4-AP (100 μM) at various times (between 15 and 45 min). In the absence of PAR peptide, only weak ERK2 and p38 phosphorylation was observed in adherent platelets (Fig. 4B). The addition of PAR1-AP and PAR4-AP induced strong and similar ERK2 and p38 phosphorylation. Altogether, these results show that ERK2 and p38 are required for platelet spreading but that another pathway is likely to mediate the differential response to PAR peptides in platelet spreading.

Sustained Ca\textsuperscript{2+} Mobilization Required for Platelet Spreading—To further assess the potential ability of PAR-APs to transduce full spreading, we examined the role and mobilization of Ca\textsuperscript{2+} in platelets bound to fibrinogen. First, we confirmed that Ca\textsuperscript{2+} was essential for platelet spreading. Platelets were allowed to sediment on fibrinogen. After 30 min, non-adherent platelets were removed, and PAR-APs were added to the adherent platelets in the presence or absence of BAPTA-1/AM (10 μM). The addition of BAPTA-1/AM impaired platelet spreading induced by PAR-APs, confirming the central role of Ca\textsuperscript{2+}. Next, to test the mobilization of Ca\textsuperscript{2+} during spreading, platelets were loaded with the Ca\textsuperscript{2+} reporter dye Oregon Green 488 BAPTA-1/AM, and pre-adherent platelets were challenged with PAR-APs. PAR1-AP induced transient Ca\textsuperscript{2+} mobilization, which returned to the basal level after 100 s (Fig. 5B). In contrast, PAR4-AP induced increased spreading by 90 and 66%, respectively, compared with the control in the absence of peptide, whereas SB203580 inhibited platelet spreading induced by PAR1-AP and PAR4-AP by 70 and 58%, respectively. We checked that the phosphorylation levels of ERK2 and Hsp27, an indirect substrate of p38, were inhibited in the presence of U0126 and SB203580, respectively. In parallel, the phosphorylation levels of p38 and ERK2 were examined with and without PAR1-AP (50 μM) and PAR4-AP (100 μM) at various times (between 15 and 45 min). In the absence of PAR peptide, only weak ERK2 and p38 phosphorylation was observed in adherent platelets (Fig. 4B). The addition of PAR1-AP and PAR4-AP induced strong and similar ERK2 and p38 phosphorylation. Altogether, these results show that ERK2 and p38 are required for platelet spreading but that another pathway is likely to mediate the differential response to PAR peptides in platelet spreading.

**Sustained Ca\textsuperscript{2+} Mobilization Required for Platelet Spreading**—To further assess the potential ability of PAR-APs to transduce full spreading, we examined the role and mobilization of Ca\textsuperscript{2+} in platelets bound to fibrinogen. First, we confirmed that Ca\textsuperscript{2+} was essential for platelet spreading. Platelets were allowed to sediment on fibrinogen. After 30 min, non-adherent platelets were removed, and PAR-APs were added to the adherent platelets in the presence or absence of BAPTA-1/AM (10 μM). The addition of BAPTA-1/AM impaired platelet spreading induced by PAR-APs, confirming the central role of Ca\textsuperscript{2+}. Next, to test the mobilization of Ca\textsuperscript{2+} during spreading, platelets were loaded with the Ca\textsuperscript{2+} reporter dye Oregon Green 488 BAPTA-1/AM, and pre-adherent platelets were challenged with PAR-APs. PAR1-AP induced transient Ca\textsuperscript{2+} mobilization, which returned to the basal level after 100 s (Fig. 5B). In contrast, PAR4-AP induced increased spreading by 90 and 66%, respectively, compared with the control in the absence of peptide, whereas SB203580 inhibited platelet spreading induced by PAR1-AP and PAR4-AP by 70 and 58%, respectively. We checked that the phosphorylation levels of ERK2 and Hsp27, an indirect substrate of p38, were inhibited in the presence of U0126 and SB203580, respectively. In parallel, the phosphorylation levels of p38 and ERK2 were examined with and without PAR1-AP (50 μM) and PAR4-AP (100 μM) at various times (between 15 and 45 min). In the absence of PAR peptide, only weak ERK2 and p38 phosphorylation was observed in adherent platelets (Fig. 4B). The addition of PAR1-AP and PAR4-AP induced strong and similar ERK2 and p38 phosphorylation. Altogether, these results show that ERK2 and p38 are required for platelet spreading but that another pathway is likely to mediate the differential response to PAR peptides in platelet spreading.

**Sustained Ca\textsuperscript{2+} Mobilization Required for Platelet Spreading**

---

**FIGURE 2.** PAR4-AP induces full platelet spreading. Platelet suspensions were stimulated with either PAR1-AP (50 μM) or PAR4-AP (100 μM) and immediately plated for 1 h in wells precoated with fibrinogen (200 μg/ml) (A) or were stimulated with thrombin (0.3 and 25 nM), PAR1-AP (50 μM), or PAR4-AP (100 μM) after PAR1 desensitization (B). Platelets were stained with Alexa Fluor 488-labeled phalloidin. The surface area was quantified as described under “Experimental Procedures.” Data are expressed as the means ± S.E. of at least three independent experiments. ***, p < 0.001; ****, p < 0.001 (statistically significant according to Student’s t test); Pret, pretreatment.

**FIGURE 3.** PAR1-AP and PAR4-AP induce a similar secretion of 5-[14C]HT. Platelet suspensions were stimulated with either PAR1-AP (50 μM) or PAR4-AP (100 μM) and immediately plated for 1 h in wells precoated with fibrinogen (200 μg/ml). Release of 5-[14C]HT was quantified in the supernatant. Data are expressed as the means ± S.E. of at least three independent experiments.
Protease-activating Receptor PAR4 and MAPKs

FIGURE 4. ERK2 and p38 MAPKs are required for platelet spreading. A (upper panel), platelet suspensions preincubated with indomethacin (5 μM) were incubated with MAPK inhibitors (U0126 (10 μM) and SB203580 (10 μM)) or Me2SO (Control) for 15 min. They were then stimulated or not with PAR1-AP (50 μM) or PAR4-AP (100 μM) and immediately plated in wells precoated with fibrinogen (200 μg/ml) for 1 h at 20 °C. The surface area was quantified as described under “Experimental Procedures.” Results are representative of three independent experiments. Data are expressed as the means ± S.E. of at least three independent experiments. ***, p < 0.001. PAR1-AP- and PAR4-AP-induced ERK2 and p38 MAPK activation. A (middle and lower panels) and B, washed human platelets were stimulated with PAR1-AP (50 μM) or PAR4-AP (100 μM) in the presence or absence of MAPK inhibitors (U0126 (10 μM) and SB203580 (10 μM)) or Me2SO for 60 min (A) or for various periods of time (15–45 min; B); ERK2, p38, and Hsp27 phosphorylation was analyzed by Western blotting using a polyclonal antibody specific to phosphorylated p38 (p38-P), Hsp27 (Hsp27-P), or ERK2 (ERK2-P) and a monoclonal antibody recognizing tubulin.

and sustained Ca2+ mobilization. In conclusion, only PAR4-AP induces full Ca2+ mobilization required for platelet spreading.

ERK2 and p38 Induced by PAR4-AP Act Downstream of ADP—The identity of the signaling pathway of MAPKs in platelet spreading induced by PAR4-AP and the role of ADP were investigated. ADP scavenging using apyrase specifically inhibited PAR4-AP-induced spreading by 66% (Fig. 6A). In addition, in the presence of apyrase, the spreading of unstimulated platelets and PAR4-induced spreading were significantly different (p < 0.001) (Fig. 6A), showing that spreading occurs via an ADP-dependent and -independent pathway. Moreover, in the presence of the MEK inhibitor U0126, a similar decrease in spreading occurred with and without apyrase (123 and 121%, respectively). In contrast, in the presence of the p38 inhibitor SB203580, a small but non-significant decrease was observed. This indicates that ERK2 and p38 are involved in platelet spreading and are coupled to the ADP-activated pathway required for PAR4-induced spreading.

We next examined the role of ERK2 and p38 in ADP secretion. Secreted 5-[14C]HT was increased after the addition of PAR4-AP (100 μM) compared with the control in the absence of peptide (Fig. 6B). Moreover, U0126 and SB203580 had no effect on 5-[14C]HT secretion induced by PAR4-AP. Because ADP is co-secreted with 5-HT upon platelet stimulation, our data suggest that MAP kinases act downstream of ADP.

To confirm ADP dependence, ERK2 and p38 phosphorylation was examined in the presence or absence of apyrase. In the absence of PAR4-AP, the weak phosphorylation levels ERK2 and p38 on adherent platelets were dependent on ADP (Fig. 6C). After PAR4-AP stimulation, p38 and ERK2 phosphorylation was strongly impaired by apyrase, showing that p38 and ERK2 phosphorylation is essentially dependent on secreted ADP. Moreover, we used two antagonists (AR-C69931MX (10 μM) and MRS 2179 (10 μM)) of P2Y12 and P2Y1, the respective receptors of ADP. p38 phosphorylation was strongly impaired by AR-C69931MX, whereas ERK2 phosphorylation was inhibited by both AR-C69931MX and MRS 2179 (Fig. 6C). These results show that PAR4 activation induces ERK2 and p38 phosphorylation via an ADP- and mostly P2Y12-dependent pathway.

ERK2 and p38 Activation: Involvement of Outside-in Signaling through Integrin αIIbβ3—Because ERK2 and p38 were involved in platelet spreading and not adhesion (data not shown), we next investigated the role of ERK2 and p38 in inside-out and outside-in signaling through integrin αIIbβ3. First, we confirmed that our adhesion assay was dependent upon αIIbβ3 engagement whether in the presence or absence of PAR4-AP because adhesion was totally inhibited by the αIIbβ3-specific recombinant antibody abciximab (Fig. 7A). Moreover, staining of the αIIbβ3 antibody PAC1, which recognizes only the active form of integrin, was observed in the presence of MAPK inhibitors (Fig. 7B). Quantification by flow cytometric analysis showed that binding of PAC1 was unaffected by MAPK inhibitors. Our results confirm that MAPKs are not involved in αIIbβ3 inside-out signaling.

To test the hypothesis that MAPKs may be involved in outside-in signaling through integrin αIIbβ3, platelets preincubated with indomethacin (5 μM) were stimulated or not with PAR4-AP (100 μM) and then deposited onto a BSA or fibrinogen matrix. In platelets on BSA, PAR4-AP induced p38 phosphorylation. This p38 phosphorylation was slightly increased in adherent platelets on fibrinogen (Fig. 7C). In contrast, ERK2 phosphorylation was observed only in adherent platelets on
fibrinogen in the presence of the PAR4 peptide. Our results show that ERK2 is downstream of αIIbβ3 and that p38 is linked only to ADP via its P2Y12 receptor.

p38 (but Not ERK2) Is Required for Actin Polymerization—Platelet spreading requires actin polymerization, which has been shown to be regulated by p38 in proliferative cells (40). We therefore compared the roles of p38 and ERK2 in actin polymerization induced by PAR4-AP. Actin polymerization induced by PAR4-AP (100 μM; 256 ± 8% of the control) was impaired by the presence of SB203580 (115 ± 3%), whereas U0126 had no effect (239 ± 4%) (Fig. 8). Our results show that actin polymerization is completely dependent on p38.

ERK2 Is Required for MLC Phosphorylation via the Rho Pathway—A previous study has shown that ERK2 is required for MLC phosphorylation (35). We therefore examined the phosphorylation status of MLC in platelet spreading. Platelets on BSA or fibrinogen were stimulated with PAR4-AP. In non-adherent platelets, i.e. in suspension, only a basal level of MLC phosphorylation was observed in the presence or absence of the PAR4 peptide (Fig. 9A). In contrast, in adherent platelets on fibrinogen, the induction of MLC phosphorylation observed in the presence of the PAR4 peptide was inhibited by U0126, showing that MLC phosphorylation induced by PAR4 is dependent on ERK2.

The role of the Rho pathway in platelet spreading and ERK2 and MLC phosphorylation has been described previously (36). Platelets were preincubated with Y-27632 (10 μM), an inhibitor of Rho kinase, and/or U0126 (10 μM) or SB203580 (10 μM) before the addition of PAR4-AP on a BSA or fibrinogen matrix. In unstimulated platelets, platelet spreading was similar in the presence or absence of the inhibitors (Fig. 9B). In contrast, when induced by PAR4-AP, platelet spreading was reduced by 63 and 46% with Y-27632 and U0126, respectively. The inhibitory effect was not additive because, when used together, the inhibitors did not lead to enhanced inhibition (60%), suggesting that Rho kinase and ERK2 belong to the same pathway. To confirm these results, we examined the phosphorylation status of ERK2, p38, and MLC in the presence of the Rho kinase inhibitor Y-27632 in a platelet suspension on BSA or in adherent platelets on fibrinogen (Fig. 9C). In contrast to p38 phosphorylation, ERK2 phosphorylation was inhibited by Y-27632 (10 μM) (Fig. 9C). Moreover, MLC phosphorylation induced by PAR4-AP returned to the basal level when U0126 and Y-27632 were added, whereas SB203580 had no effect (data not shown). Together, these results show that ERK2, involved in the outside-in signaling of integrin αIIbβ3, is dependent on the Rho pathway and participates in cytoskeleton rearrangement.

DISCUSSION

After thrombin induction in human platelets, PAR1 and PAR4 mediate signaling with different activation kinetics (12, 37). PAR1 is a high affinity thrombin receptor, and PAR4 is a low affinity receptor that is activated more slowly (12, 37). The difference in PAR1 and PAR4 signaling kinetics implies that the two PARs may play distinct roles in early and late platelet activation events. We therefore investigated platelet adhesion and spreading on a fibrinogen matrix. PAR1 and PAR4 stimulation induced identical adhesion,
whereas only PAR4 stimulation induced full spreading on a fibrinogen matrix. Moreover, low concentrations of thrombin, which essentially activates PAR1, increased spreading, as did specific PAR1 stimulation. At higher thrombin concentrations involving PAR1 and PAR4, spreading was more intense, reinforcing the results obtained with PAR-APs and indicating that PAR4 activation is required for full spreading. As ADP is known to play a critical role in the thrombin-induced signaling pathway, we investigated whether secretion of ADP could explain the difference observed between PAR-APs. Although ADP was necessary for platelet adhesion (data not shown) and spreading, PAR4-AP did not induce higher secretion compared with PAR1-AP, showing that secreted ADP is required but not sufficient for full spreading and that a concomitant signal through PARs is involved. Ca$^{2+}$ mobilization required as a second messenger for platelet adhesion and spreading could be a candidate because, in contrast to PAR4-AP, which induced prolonged Ca$^{2+}$ mobilization, PAR1-AP has been reported to induce only transient Ca$^{2+}$ release (12). In fact, we confirmed that platelet spreading in the presence or absence of PAR peptides required Ca$^{2+}$ and that only PAR4-AP induced sustained Ca$^{2+}$ mobilization during spreading. This discrepancy observed regarding Ca$^{2+}$ between thrombin receptors was reinforced by a recent study showing that PAR4 (but not PAR1) induces sustained Ca$^{2+}$ mobilization (38). Signaling through MAPKs regulates adhesion and spreading in proliferative cells (16). We have shown previously a complementary effect of ERK2 and p38 in the shear stress-dependent control of platelet adhesion to collagen (39). Here, we have described a complementary role of ERK2 and p38 in platelet spreading induced by PAR4-AP without any effect on adhesion to fibrinogen (data not shown) and integrin αIIbβ3 activation, suggesting that MAPKs act in post-occupancy events required for spreading. In fact, p38 was dependent on ADP via its P2Y12 receptor. Moreover, p38 induced polymerization of actin required for lamellipodium formation, as described previously in proliferative cells (40). This finding is in agreement with literature showing that the p38 inhibitor does not affect the increase in platelet adhesion to fibrinogen induced by a low dose of thrombin, in contrast to platelet adhesion to fibrinogen induced by the thromboxane A$_2$ analog and by 8-isoprostaglandin F$_2α$ (30). It was suggested that the thromboxane A$_2$ analog does not enhance the affinity/avidity of αIIbβ3 and does not induce up-regulation of intracellularly stored proteins (30). Moreover, mouse p38α$^{+/−}$ platelets activated by the thromboxane A$_2$ analog bind poorly to fibrinogen compared with mouse wild-type platelets (41). After artery carotid injury, thrombotic occlusion is longer in p38α$^{+/−}$ absence of apyrase (7.5 units/ml) for 15 min. They were then stimulated or not with PAR4-AP (100 μM) and immediately plated in wells precoated with fibrinogen (200 μg/ml) for 1 h at 20°C. A, platelet spreading was quantified as described under “Experimental Procedures.” Results are representative of four different experiments. Data are expressed as the means ± S.E. of at least three independent experiments. **, *p < 0.01; ***, *p < 0.001 (statistically significant according to Student’s t test). B, release of 5-[14C]HT was quantified in the supernatant. C, p38 (p38-P) and ERK2 (ERK2-P) phosphorylation was analyzed after platelet treatment with apyrase, AR-C69931MX (10 μM), or MRS 2179 (10 μM).
mice than in wild-type mice (41). These data strongly support the involvement of p38 in thrombus formation.

In contrast to p38, ERK2 activation was dependent on integrin αIIbβ3, and the involvement of ADP in ERK2 activation was a consequence of the role of ADP in the activation of αIIbβ3. Our results are in agreement with our previously published data showing that, in platelet aggregation, ADP alone is unable to induce ERK2 activation, whereas secreted ADP following stimulation with collagen or thrombin is involved in ERK2 activation (23). ERK2 controlled MLC phosphorylation induced by PAR4-AP, and phosphorylation of both ERK2 and MLC was dependent on αIIbβ3 and the Rho pathway. Moreover, the Rho pathway was involved in platelet spreading, but did not affect platelet adhesion (data not shown). The link between ERK2 and MLC phosphorylation is still unknown. The hypothesis concerning the requirement of intact cytoskeletal structures leading to recruitment of signaling molecules vital for ERK2 activation is consistent with our observation that cytochalasin D blocked integrin-mediated ERK2 activation (data not shown). The other hypothesis is that Rho activation is associated with MLC phosphorylation, which might result from activation of MLC kinase downstream of Rho and ERK2 (42). In this case, MLC kinase, a substrate of ERK2, phosphorylated MLC. This hypothesis can be excluded because ML7, an inhibitor of MLC kinase, did not affect MLC phosphorylation (data not shown). Finally, we cannot completely exclude the hypothesis that ERK2 might regulate MLC phosphatase activity by phosphorylation of MYPT-1 (myosin phosphatase target protein-1) (43) and consequently increase MLC phosphorylation (43). Our data are consistent

**FIGURE 7.** Outside-in signaling of integrin αIIbβ3 is required for ERK2 (but not p38 MAPK) activation. A, platelets preincubated with abciximab in the presence or absence of PAR4-AP were plated on fibrinogen for 1 h. Platelet adhesion was quantified as described under “Experimental Procedures.” Data are expressed as the means ± S.E. of at least three independent experiments. ***, p < 0.01 (statistically significant according to Student’s t test). B, platelet suspensions with indomethacin (5 μM) were preincubated with MAPK inhibitors (U0126 (10 μM) and SB203580 (10 μM)) or MeSO, and staining of PAC1 was analyzed by immunofluorescence microscopy and flow cytometric analysis. C, platelet suspensions with indomethacin (5 μM) were preincubated with PAR4-AP (100 μM) and immediately plated in wells precoated with BSA and fibrinogen (Fg; 200 μg/ml) for 1 h at 20 °C. p38 (p38-P) and ERK2 (ERK2-P) phosphorylation was analyzed by Western blotting.
with a role of ERK2 in post-occupancy events required for spreading. Finally, the fact that RhoA did not inhibit p38 phosphorylation and that the p38 inhibitor did not affect ERK2 or MLC phosphorylation strongly confirms that ERK2 and p38 act on platelet cytoskeleton reorganization through two different pathways.

Together, our results show a new physiological significance of PAR4 activation, with a major role for PAR4 in platelet spreading. We have proposed a model for how PAR4 induces platelet spreading (Fig. 10). In this model, PAR4 induces secretion of ADP. Via its P2Y12 receptor, secreted ADP induces activation of p38 and integrin αIIbβ3. Activated αIIbβ3 induces ERK2 phosphorylation via the Rho pathway. Both MAPKs act on cytoskeleton rearrangement and platelet spreading, respectively. Our data identify a new role for PAR4 at sites of high thrombin production required for the stabilization of thrombi.

Acknowledgments—We thank Dr. J. P. Rosa for helpful discussion. We are indebted to Drs. C. Pouzet and E. Sulpice for technical assistance with confocal microscopy and flow cytometric analysis, respectively.

REFERENCES
1. Okumura, T., Hasitz, M., and Jamieson, G. A. (1978) J. Biol. Chem. 253, 3435–3443
2. Jamieson, G. A., and Okumara, T. (1978) J. Clin. Investig. 61, 861–864
3. Ganguly, P., and Gould, N. L. (1979) Br. J. Haematol. 42, 137–145
4. De Marco, L., Mazzucato, M., Masotti, A., Fenton, J. W., and Ruggeri, Z. M. (1991) J. Biol. Chem. 266, 23776–23783
5. Vu, T.-K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 1057–1068
6. Kahn, M. L., Zheng, Y. W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, R. V., Jr., Tam, C., and Coughlin, S. R. (1998) Nature 394, 690–694
7. Xu, W. F., Andersen, H., Whitmore, T. E., Pressnell, S. R., Yee, D. P., Ching, A., Gilbert, T., Davie, E. W., and Foster, D. C. (1998) Proc. Natl. Acad. Sci.
8. Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H., and Coughlin, S. R. (1999) *J. Clin. Investig.* **103**, 879–887
9. Vu, T.-K. H., Wheaton, V. I., Hung, D. T., Charo, I., and Coughlin, S. R. (1991) *Nature* **353**, 674–677
10. Hung, D. T., Wong, Y. H., Vu, T.-K., and Coughlin, S. R. (1992) *J. Biol. Chem.* **267**, 20831–20834
11. Lau, L. F., Pumiglia, K., Cote, Y. P., and Feinstein, M. B. (1994) *Biochem. J.* **303**, 391–400
12. Covic, L., Gresser, A. L., and Kuliopulos, A. (2000) *Biochemistry* **39**, 5458–5467
13. Aharonovitz, O., and Granot, Y. (1996) *J. Biol. Chem.* **271**, 16494–16499
14. Kramer, R. M., Roberts, E. F., Strifler, B. A., and Johnstone, E. M. (1995) *J. Biol. Chem.* **270**, 27395–27398
15. Rossomando, A. J., Payne, D. M., Weber, M. J., and Sturgill, T. W. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 6940–6943
16. Lau, L. F., Pumiglia, K., Cote, Y. P., and Feinstein, M. B. (1994) *Biochem. J.* **303**, 391–400
17. Covic, L., Gresser, A. L., and Kuliopulos, A. (2000) *Biochemistry* **39**, 5458–5467
18. Aharonovitz, O., and Granot, Y. (1996) *J. Biol. Chem.* **271**, 16494–16499
19. Kramer, R. M., Roberts, E. F., Strifler, B. A., and Johnstone, E. M. (1995) *J. Biol. Chem.* **270**, 27395–27398
20. Rossomando, A. J., Payne, D. M., Weber, M. J., and Sturgill, T. W. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 6940–6943
21. Lau, L. F., Pumiglia, K., Cote, Y. P., and Feinstein, M. B. (1994) *Biochem. J.* **303**, 391–400
22. Covic, L., Gresser, A. L., and Kuliopulos, A. (2000) *Biochemistry* **39**, 5458–5467
23. Aharonovitz, O., and Granot, Y. (1996) *J. Biol. Chem.* **271**, 16494–16499
14. Kramer, R. M., Roberts, E. F., Strifler, B. A., and Johnstone, E. M. (1995) *J. Biol. Chem.* **270**, 27395–27398
15. Rossomando, A. J., Payne, D. M., Weber, M. J., and Sturgill, T. W. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 6940–6943
16. Lau, L. F., Pumiglia, K., Cote, Y. P., and Feinstein, M. B. (1994) *Biochem. J.* **303**, 391–400
22. Covic, L., Gresser, A. L., and Kuliopulos, A. (2000) *Biochemistry* **39**, 5458–5467