Platelet ERK2 activation by thrombin is dependent on calcium and conventional protein kinases C but not Raf-1 or B-Raf

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Abstract **Extracellular signal-regulated kinase (ERK) activation pathways have been well characterized in a number of cell types but very few data are available for platelets. The thrombin-induced signaling pathway leading to ERK2 activation in platelets is largely uncharacterized. In this study, we investigated the kinases involved in thrombin-induced ERK2 activation in conditions of maximal ERK2 activation. We found that thrombin-induced mitogen-activated protein kinase/ERK kinase (MEK)1/2 activation was necessary for ERK2 phosphorylation. We obtained strong evidence that conventional protein kinase Cs (PKCs) and calcium are involved in thrombin-induced ERK2 activation. First, ERK2 and MEK1/2 phosphorylation was totally inhibited by low concentrations (1 μM) of RO318425, a specific inhibitor of conventional PKCs. Second, Ca^{2+}, from either intracellular pools or the extracellular medium, was necessary for ERK2 activation and conventional PKC activation, excluding the involvement of a new class of calcium-insensitive PKCs. Third, LY294002 and wortmannin had no significant effect on ERK2 activation, even at concentrations that inhibit phosphatidylinositol (PI)3-kinase (5 μM to 25 μM and 50 nM, respectively). This suggests that PI3-kinase was not necessary for ERK2 activation and therefore, that PI3-kinase-dependent atypical PKCs were not involved. Surprisingly, in contrast to proliferative cells, we found that the serine/threonine kinases Raf-1 and B-Raf were not an intermediate kinase between conventional PKCs and MEK1/2. After immunoprecipitation of Raf-1 and B-Raf, the basal glutathione *S*-transferase–MEK1 phosphorylation observed in resting platelets was not upregulated by thrombin and was still observed in the absence of anti-Raf-1 or anti-B-Raf antibodies. In these conditions, the in vitro cascade kinase assay did not detect any MEK activity. Thus in platelets, thrombin-induced ERK2 activation is activated by conventional PKCs independently of Raf-1 and B-Raf activation.**

Key words: Platelet; Extracellular signal-regulated kinase-2; Conventional protein kinase C; Raf

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; JNK, C-Jun N-terminal kinase; PKC, protein kinase C; RGDS, Arg-Gly-Asp-Ser; PMA, phorbol myristate acetate; TPO, thrombopoietin

1. Introduction

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases [1] divided into three subgroups: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK ($p38^{MAPK}$). The signaling pathways and the role of MAPKs, especially ERKs, have been extensively investigated in proliferative cells [2]. Briefly, ERKs may be activated by growth factors and hormones with tyrosine kinase receptors [3], G protein-coupled receptors [4] and by integrins [5]. The signaling pathway first induces activation of the small G protein Ras by conversion from an GDP-bound to a GTP-bound state [6]. This is followed by interaction between Ras and the serine/threonine kinase Raf-1. Raf-1 phosphorylation leads to activation of the dual tyrosine/threonine-specific MAPK/ERK kinase (MEK)1 and MEK2 [7], which in turn phosphorylate and activate ERK1 and ERK2 at threonine and tyrosine residues [8]. Active ERKs elicit many of the effects of extracellular signals, through the regulation and phosphorylation of many substrates, including phospholipase A2 [9] and $p90^{rsk}$ [10] and nuclear proteins, such as $p62^{TCF}$ or Elk-1 [11], leading to proliferation [12], differentiation [13] and adhesion [14].

In contrast, the signal transduction mechanisms leading to the activation of MAPKs in platelets are largely uncharacterized. ERK1, ERK2 and $p38^{MAPK}$ have been detected in platelets. [15,16]. Recently, we found that JNK1 is also present in human platelets and activated after thrombin induction [17]. The members of all three families (ERK2, $p38^{MAPK}$ and JNK1) are activated by physiologic agonists including thrombin [18] and collagen [19]. ERK2 activation is mediated by the dual-specificity kinase MEK1/2 [20] and seems to involve a protein kinase C (PKC)-dependent pathway [21]. H-Ras [22] and Raf-1 [23] have been shown to be present in platelets, but the relationships between the upstream kinases involved in the activation of ERK2 are unknown. Finally, we have found that in human platelets, the activation of ERK2 and JNK1 by thrombin is negatively regulated by αIIbβ3 engagement in aggregation [24,17]. These reports were the first to show the downregulation of MAPKs (ERK2 and JNK1) by an integrin.

In this study, we investigated the putative kinases involved in thrombin-induced ERK2 activation. We found that thrombin-induced ERK2 activation was dependent on both calcium and conventional PKCs but independent of Raf-1 and B-Raf.

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2. Materials and methods

2.1. Reagents

Bovine α-thrombin, the synthetic peptide Arg-Gly-Asp-Ser (RGDS), myelin basic protein (MBP), leupeptin and aprotinin were purchased from Sigma (St. Louis, MO, USA). RO318425, GF109203X, wortmannin and LY294002 were purchased from Calbiochem (Meudon, France). BAPTA-AM and PD98059 were from Biomol (Plymouth Meeting, PA, USA). Rabbit polyclonal antibodies raised against the C-terminal peptides of ERK1/2 (C-14), Raf-1 (C-12) and B-Raf (C-19) and monoclonal antibody directed against PKC(s) (MC5) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody directed against the phosphorylated forms of ERKs (ERKs-P) was obtained from Promega (Madison, WI, USA. Rabbit polyclonal antibodies directed against the phosphorylated form of MEK1/2 (MEK1/2-P) and Akt (Akt-P) were purchased from New England Biolabs (Beverly, MA, USA). Inactive GST–MEK1 was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Donkey anti-rabbit horseradish peroxidase-conjugated IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and [γ-^{32}P]ATP (167 TBq/mmol) from ICN (Irvine, CA, USA). A Raf immunoprecipitation kinase cascade assay kit was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Thrombopoietin (TPO) mimetic peptide was a generous gift from Dr. A. Dubart (Paris, France).

2.2. Platelet preparation and aggregation

Venous blood was collected from healthy donors free of medication for at least 2 weeks. Platelet-rich plasma was obtained by centrifugation of whole blood at $120\times g$ for 15 min at 21°C, and platelets were isolated by differential centrifugation, in citrate buffer, pH 6, containing 10^{-4} mM prostaglandin E_1, 140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, and 12.5 mM sucrose, and then in the same buffer but without prostaglandin E_1. The platelet pellet was resuspended in 10 mM HEPES, pH 7.4, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$, and 10 mM glucose. Cell concentration was adjusted to 5×10^8/ml. Platelets (0.4 ml) were preincubated at 37°C, without stirring, for various times, with various molecules. Platelet aggregation was then initiated by adding bovine thrombin (1 NIH U/ml), with constant stirring (1200 rpm) in an aggregometer cuvette (Chronolog dual beam aggregometer). Aggregation was measured and expressed as a percent change in the transmission of light, with the blank sample (buffer without platelets) defined as 100%.

2.3. Immunoblotting

Samples were subjected to immunoblotting as described previously [24]. Briefly, platelet lysates were obtained by adding sodium dodecyl sulfate (SDS) denaturing buffer and heating the samples at 95°C for 5 min. Proteins were subjected to 12% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filters by semi-dry transfer (Enprotech, Natick, MA, USA). Filters were then incubated for 1 h at room temperature with the primary polyclonal antibody: anti-ERKs (1:20 000), anti-ERKs-P (1:20 000), anti-MEK1/2-P (1:500), anti-Akt-P (1:1000), anti-Raf-1 (1:1000) and anti-B-Raf (1:1000). Membranes were washed five times with phosphate-buffered saline, without milk. They were then incubated with either horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20 000 or 1:10 000) or with peroxidase-conjugated rabbit anti-mouse IgG (1:10 000) for 45 min at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce).

2.4. Raf kinase assay

Immunoprecipitation and in vitro kinase assay for Raf-1 and B-Raf: platelet lysates were obtained by adding 40 μl of lysis buffer (10% Triton, 500 μM NaF, 100 mM β-glycerophosphate, pH 7.5). Platelet lysates (800 μl) were incubated overnight at 4°C with the polyclonal anti-Raf-1 antibody (2 μg/sample) or anti-B-Raf (3 μg/sample) and then with protein-A or protein-G Sepharose beads for 2 h at 4°C. Immunoprecipitates were washed three times in buffer A (0.2% Triton, 10 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 40 mM β-glycerophosphate, 0.2 mM orthovanadate, 5 μg/ml leupeptin, 10 μg/ml aprotinin), once in buffer B (buffer A supplemented with 500 mM NaCl), once in buffer A, once in buffer C (10 mM Tris, 40 mM β-glycerophosphate, 0.2 mM orthovanadate, 5 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.2) and once in kinase buffer (20 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$). The samples were then resuspended in 20 μl of kinase buffer supplemented with inactive GST–MEK1 (1 μg) and with 10 μCi of [γ-^{32}P]ATP and 1 μM of unlabeled ATP. After 30 min at 30°C, the reaction was stopped by adding Laemmli buffer, and the samples were subjected to 10% SDS–PAGE. The gel was then stained, dried and subjected to autoradiography and phosphorylated GST–MEK was analyzed.

Culture and cell treatment of human megakaryoblastic UT7-Mpl expressing the full-length murine TPO receptor Mpl was processed as previously described [25].

Raf activity was measured according to the manufacturer's instructions. Briefly, after immunoprecipitation of Raf-1 or B-Raf, the in vitro kinase cascade reaction was initiated by adding inactive GST–MEK1 and inactive GST–ERK. MBP was added as a substrate with [γ-^{32}P]ATP. The reaction mixtures were spotted onto phosphocellulose membranes. After several washings, the radioactivity of each spot, corresponding to phosphorylated MBP (MBP-^{32}P), was quantified.

3. Results

3.1. MEK1/2 activation is necessary for thrombin-induced ERK2 activation

We have shown that platelet ERK2 is activated by α-thrombin [24]. To identify the signaling pathway involved in thrombin-induced ERK2 activation, we first investigated the involvement of MEK1/2, the tyrosine/threonine kinase directly responsible for ERK2 activation, in conditions of maximal ERK2 activation. We used thrombin activation in the presence of RGDS peptide, which completely blocks αIIbβ3 integrin engagement and as we have previously demonstrated, upregulates ERK2 activity. We first compared the kinetics of ERK2 and MEK1/2 activation with and without PD98059, a specific inhibitor of MEK1/2. Western blotting was performed, using an antibody recognizing only the phosphorylated forms of ERK2 (ERK2-P), MEK1/2 (MEK1/2-P) (Fig. 1A,B) or total ERK2 (Fig. 1C). The phosphorylation of

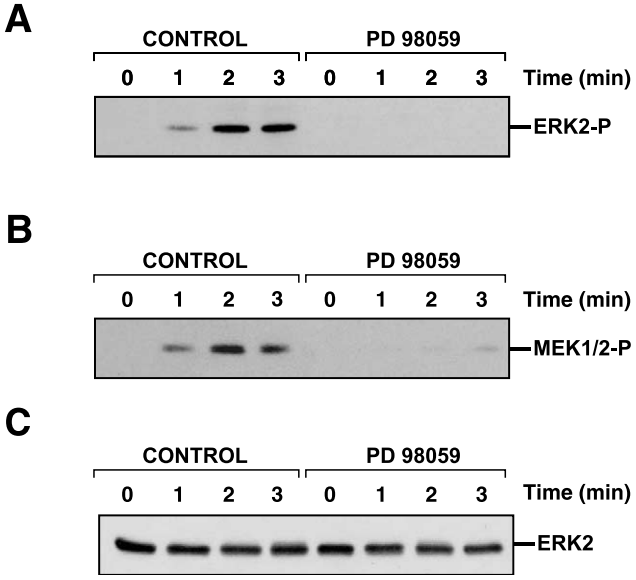

Fig. 1. Effect of PD98059 on the phosphorylation of ERK2 and MEK1/2. Washed platelets were preincubated with stirring, for 1 min, with or without PD98059 (20 μM) and were then stimulated with thrombin (1 NIH U/ml) in the presence of the RGDS peptide (1 mM) for various times, with stirring. Platelet lysates were analyzed by SDS–PAGE followed by Western blotting using antibodies recognizing ERK2-P (A), MEK1/2-P (B) and total ERK2 (C). Results are representative of four experiments.

ERK2 and MEK1/2 was detected after 1 min of thrombin stimulation and peaked at 3 min. Preincubation with PD98059 (20 μM) for 1 min inhibited the activation of ERK2 and MEK1/2 (Fig. 1A,B). These results confirm that MEK1/2 activates ERK2 in human platelets.

3.2. Thrombin-induced ERK2 activation is dependent on calcium and conventional PKCs

We next investigated whether PKCs, a serine/threonine kinase, could activate MEK1/2 and consequently ERK2, as previously demonstrated in other cell systems such as COS cells [26], and in angiotensin II-induced vascular smooth muscle cell activation [27]. Thrombin-induced ERK2 and MEK1/2 activation was assessed in the presence and absence of a selective inhibitor of PKC, RO318425, which is known to inhibit conventional PKCs [28]. Platelets were incubated with various concentrations of RO318425 (0–10 μM) for 2 min with stirring. Activation, as assessed by Western blotting, was inhibited by 90% for ERK2 and 100% for MEK1/2 by 1 μM of RO318425 (Fig. 2A). Consistent results were obtained in kinetic studies in which the activation of ERK2 and MEK1/2 was blocked at all time points (Fig. 2B). Similar results were also obtained with another specific inhibitor of PKCs, GF109203X (data not shown). To confirm the involvement of PKCs, we investigated the effect of phorbol myristate acetate (PMA) on the activation of MEK1/2 and ERK2. PMA (500 nM) induced the activation of MEK1/2 and ERK2. Activation became detectable after 1–2 min, depending on the experiment and reached a maximum between 2 and 3 min (Fig. 2C). These results suggest that thrombin-induced MEK1/2 activation and subsequent ERK2 activation, are mediated by a conventional PKC-dependent pathway.

Conventional PKC isoenzymes are activated via a calcium-dependent mechanism. We investigated the putative role of calcium in the pathway of ERK2 activation induced by thrombin. Platelets were resuspended in the presence or ab-

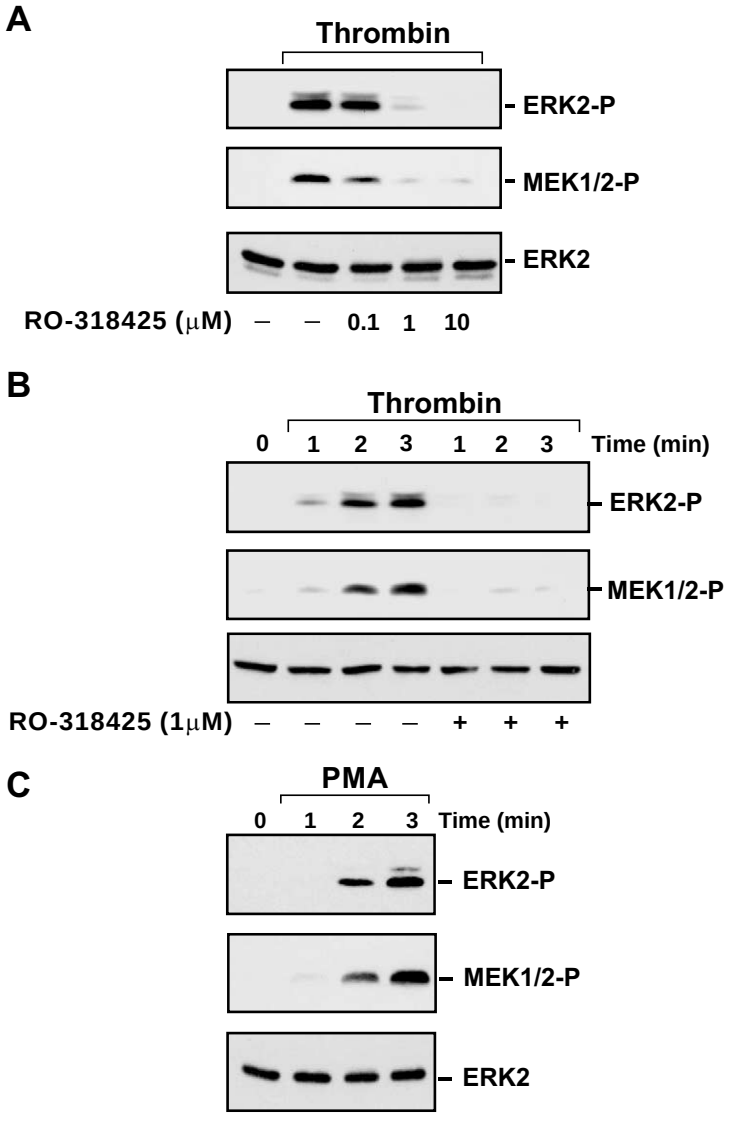

Fig. 2. Effect of PKCs on the activation of MEK1/2 and ERK2. Washed platelets were preincubated without stirring for 10 min with various concentrations of RO318425 (0.1, 1 and 10 μM) (A) or with 1 μM of RO318425 (B) in the presence or absence of RGDS peptide (1 mM). They were then stimulated with thrombin (1 NIH U/ml) (A,B) with stirring for 2 min (A) or the times indicated (B) or with PMA (500 nM) (C). The platelets were solubilized in SDS buffer and analyzed for ERK2 activation by SDS–PAGE followed by Western blotting, as described in Fig. 1. Results are representative of five experiments.

sence of calcium (1 mM) and stimulated by incubation with thrombin (1 NIH U/ml) for 2 min (Fig. 3A). In these conditions, the phosphorylation of ERK2 and MEK1/2 was maximal with calcium (1 mM) in platelet suspension buffer. Densitometric analysis showed five times more ERK2 phosphorylation and three times more MEK1/2 phosphorylation in the presence of extracellular calcium than in the absence of calcium (Fig. 3A), suggesting that calcium influx may be involved in thrombin-induced ERK2 activation. The phosphorylation of ERK2 and MEK1/2 was completely inhibited if platelets were incubated in the absence of extracellular calcium and with 0.2 mM BAPTA, a chelator of intracellular calcium. Thus, the activation of MEK1/2 and ERK2 by thrombin is dependent on intracellular calcium. These results suggest a key role in the activation of MEK1/2 and ERK2 by thrombin of cytosolic calcium originating from (1) the extracellular medium and (2) the release of calcium from intracellular stores. This is consistent with a central role for calcium-dependent PKCs (the conventional class of PKCs) and seems to exclude involvement of the new class of calcium-independent PKC(s). We also investigated the role of the atypical class of PKCs, such as PKCζ, which is activated by the products of phosphatidylinositol (PI)3-kinase [29]. Wortmannin (10–50 nM) and LY294002 (5–25 μM) were used to inhibit PI3-kinase activity. Concentrations of wortmannin (50 nM) and LY294002 (5–25 μM) that inhibited Akt phosphorylation (Akt-P) had no effect on thrombin-induced ERK2 activation in the presence of RGDS peptide (Fig. 3B). Thus, PI3-kinase has no effect on ERK2 activation, suggesting that atypical PKCs are not involved in thrombin-induced ERK2 activation. Overall, these results are consistent with the mediation of thrombin-induced ERK2 activation by conventional PKCs.

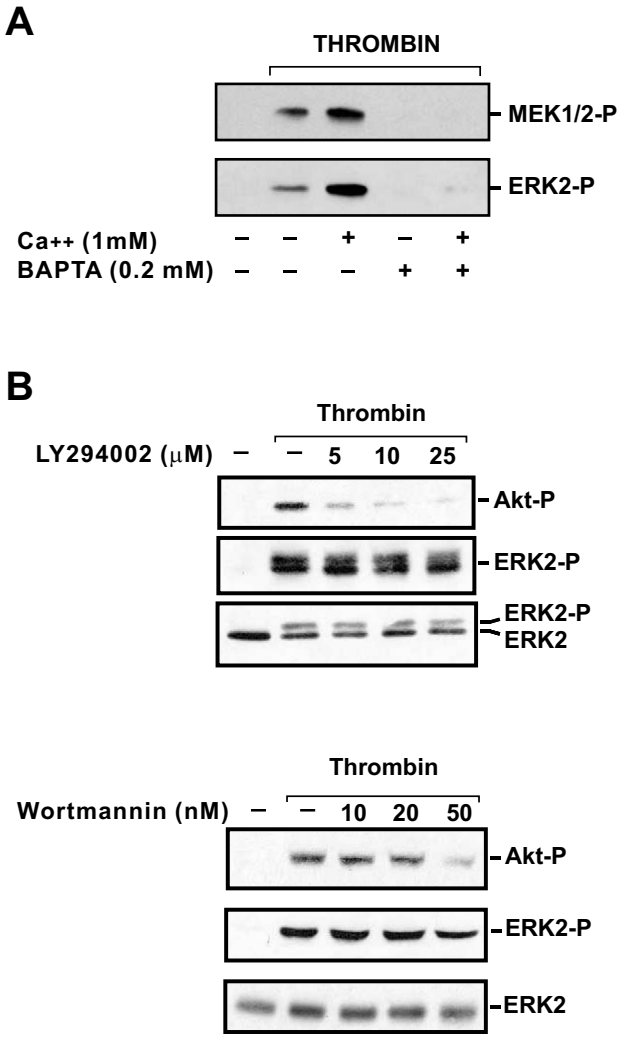

Fig. 3. Role of conventional PKCs on the phosphorylation of MEK1/2 and ERK2. A: Effect of Ca^{2+}. Washed platelets, with or without calcium (1 mM) in the extracellular medium were preincubated without stirring for 10 min in the presence or absence of BAPTA (0.2 mM). They were then stimulated by thrombin (1 NIH U/ml), with stirring for 2 min. Platelet lysates were obtained as previously described and analyzed by SDS–PAGE followed by Western blotting using polyclonal antibodies recognizing MEK1/2-P, ERK2-P or total ERK. Results are representative of five experiments. B: Effect of wortmannin and LY294002. Washed platelets were preincubated for 20 min without stirring with various concentrations of LY294002 (5–25 μM) or wortmannin (10–50 nM) in the presence of RGDS peptide (1 mM). They were then stimulated with thrombin (1 NIH U/ml). Samples were solubilized in SDS buffer and subjected to SDS–PAGE followed by Western blotting as described previously. Results are representative of five experiments.

3.3. *Raf-1 and B-Raf are not involved in thrombin-induced ERK2 activation*

We next assessed the involvement of the classical serine/threonine kinase Raf-1, which has been reported to be activated by PKCs [30] and to activate MEK1/2 directly in proliferative cells [31]. Raf-1 has been detected in platelets [23]. We therefore investigated whether it was activated by thrombin. After immunoprecipitation of Raf-1 followed by in vitro phosphorylation of GST-MEK1, basal phosphorylation of GST-MEK1, detected in resting platelets, was not increased after thrombin induction in the presence of RGDS peptide, conditions of maximal ERK2 activation (Fig. 4A). Moreover, in the absence of Raf-1 antibody, a non-specific phosphorylation of GST–MEK1 was still observed in resting platelets, suggesting that the basal phosphorylation of GST–MEK1 was independent of Raf-1 activity and that the observed phosphorylation occurs at sites that are not involved in MEK activation by kinases other than Raf-1. In contrast, in smooth muscle cells, used as a positive control, platelet-derived growth factor (PDGF) induced an activation of Raf-1 compared with quiescent cells. The apparent difference in molecular weight of the Raf-1 in platelets and that in smooth muscle cells may be due to species differences (humans versus pigs) and/or different states of Raf-1 phosphorylation as previously described [32]. Moreover, Raf-1 activity was assessed in the presence and absence of the PKC inhibitor RO318425 (10 μM), which inhibited thrombin-induced ERK2 phosphorylation. Basal phosphorylation of GST–MEK was still observed in the presence of RO318425 in resting platelets and after 2 min of thrombin stimulation (Fig. 4B) whereas thrombin-induced ERK2 phosphorylation was totally inhibited (data not shown). Finally, to investigate whether basal MEK phosphorylation (Fig. 4A) masks phosphorylation on sites leading to MEK activation, we performed a cascade assay to measure MEK activation. Thus, after immunoprecipitation of Raf-1 a cascade assay was performed, in which GST–MEK phosphorylated GST–ERK, which in turn phosphorylated a substrate such as MBP. In these conditions, a basal phosphorylation of MBP-^{32}P was observed in resting platelets and after thrombin induction. This phosphorylation

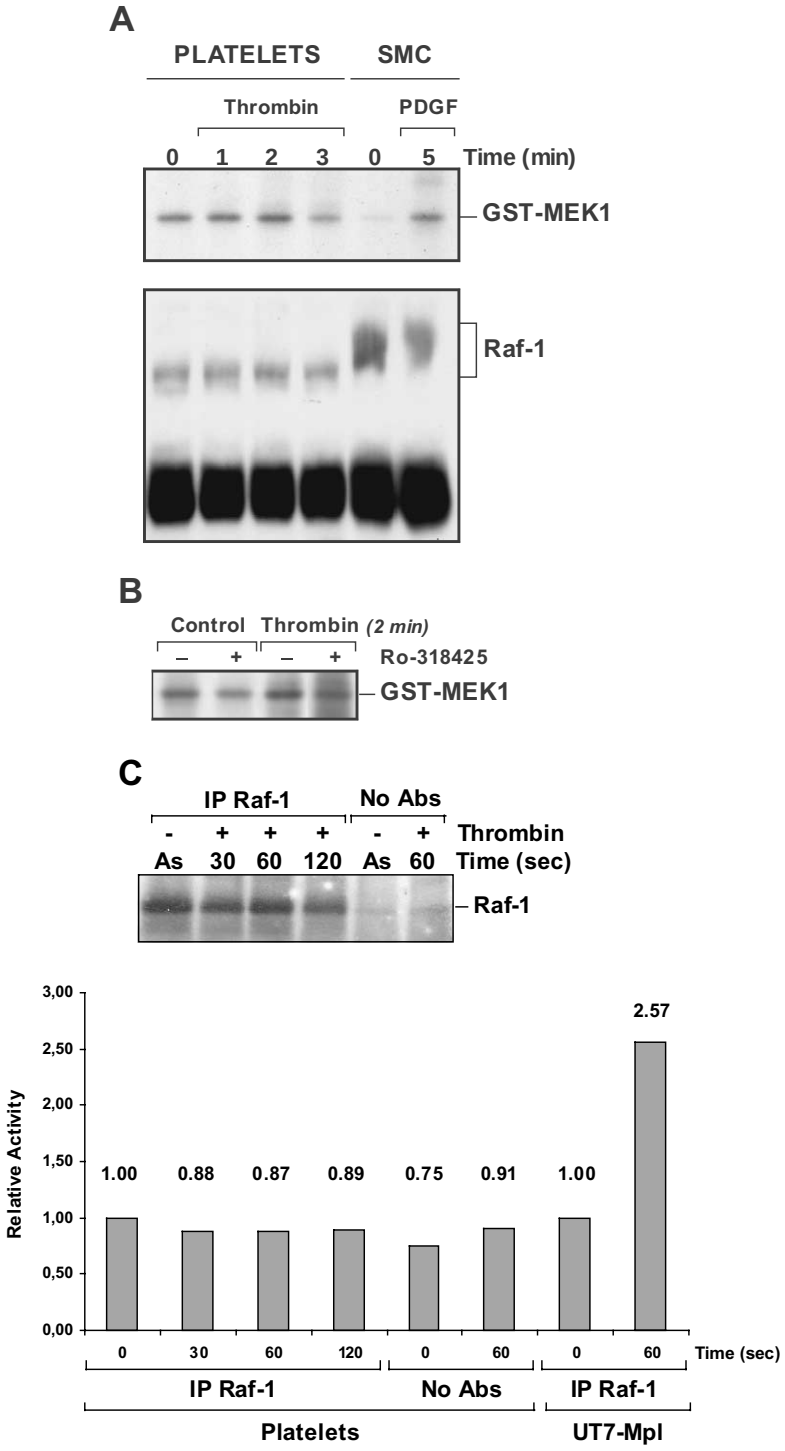

Fig. 4. Raf-1 activity in thrombin-stimulated platelets. Washed platelets were preincubated in the presence of the RGDS peptide (1 mM) and then activated by thrombin (1 NIH U/ml) (A, C) for various times or (B) with 10 μM of RO318425. Samples were solubilized in Triton buffer and then immunoprecipitated with an antibody directed against Raf-1. A,B: The exogenous substrate, GST–MEK1, was phosphorylated after the immunoprecipitation of Raf-1. C: In vitro kinase assay of MEK activation: Raf-1 was immunoprecipitated and then a cascade assay was carried out in which GST–MEK phosphorylated GST–ERK, which in turn phosphorylated MBP. Smooth muscle cells stimulated with 10 ng/ml of PDGF (5 min) and UT7-Mpl stimulated with 10 nM TPO mimetic peptide (1 h) were used as a positive control. Results are representative of three experiments.

reached the same level as in the negative control in the absence of anti-Raf1 (Fig. 4C). Thus, Raf-1 was unable to induce MEK activity in resting platelets and after thrombin induction. This strongly suggests that kinases other than Raf-1 phosphorylate MEK on sites that are not involved in MEK activation (Fig. 4A). In contrast, as a positive control, Raf-1 activity was measured in megakaryoblastic UT7-Mpl cell line stimulated with a TPO mimetic peptide. A 2.57-fold increase of immunoprecipitated Raf-1 activity was observed after 1 h of stimulation by TPO (10 nM).

All together, these data show that Raf-1 activity is not required for ERK2 activation.

We also tested another isoform of Raf, B-Raf, which is present in hematopoietic cells [33]. B-Raf was present in platelet lysate (data not shown). After immunoprecipitation of B-Raf followed by in vitro phosphorylation of GST–MEK1, a basal level of activated B-Raf was detected in resting platelets which was not upregulated by thrombin (Fig. 5A). Moreover, in the presence of the PKC inhibitor RO318427 (10 μM), the basal phosphorylation of GST–MEK was affected but still detectable (Fig. 5B). Like for Raf-1, we performed a cascade assay to measure MEK activation rather than phosphorylation. After immunoprecipitation of B-Raf, a cascade assay was performed with ATP, GST–MEK, GST–ERK and MBP. In these conditions, a basal phosphorylation of MBP-^{32}P was observed in resting platelets and after thrombin induction. This phosphorylation reached the level observed in the negative control in the absence of anti-B-Raf (Fig. 5C). B-Raf activity was still processed in TPO stimulated UT7-Mpl cell line as a positive control. We observed a 2.75-fold increase of the kinase activity after 4 h of stimulation by TPO (10 nM).

These results show that B-Raf is not involved in thrombin-induced MEK activation and suggest that kinases other than B-Raf phosphorylate MEK on sites (Fig. 5A) that are not involved in MEK activation. All these results suggest that B-Raf and Raf-1 are not involved in MEK/ERK activation after thrombin stimulation.

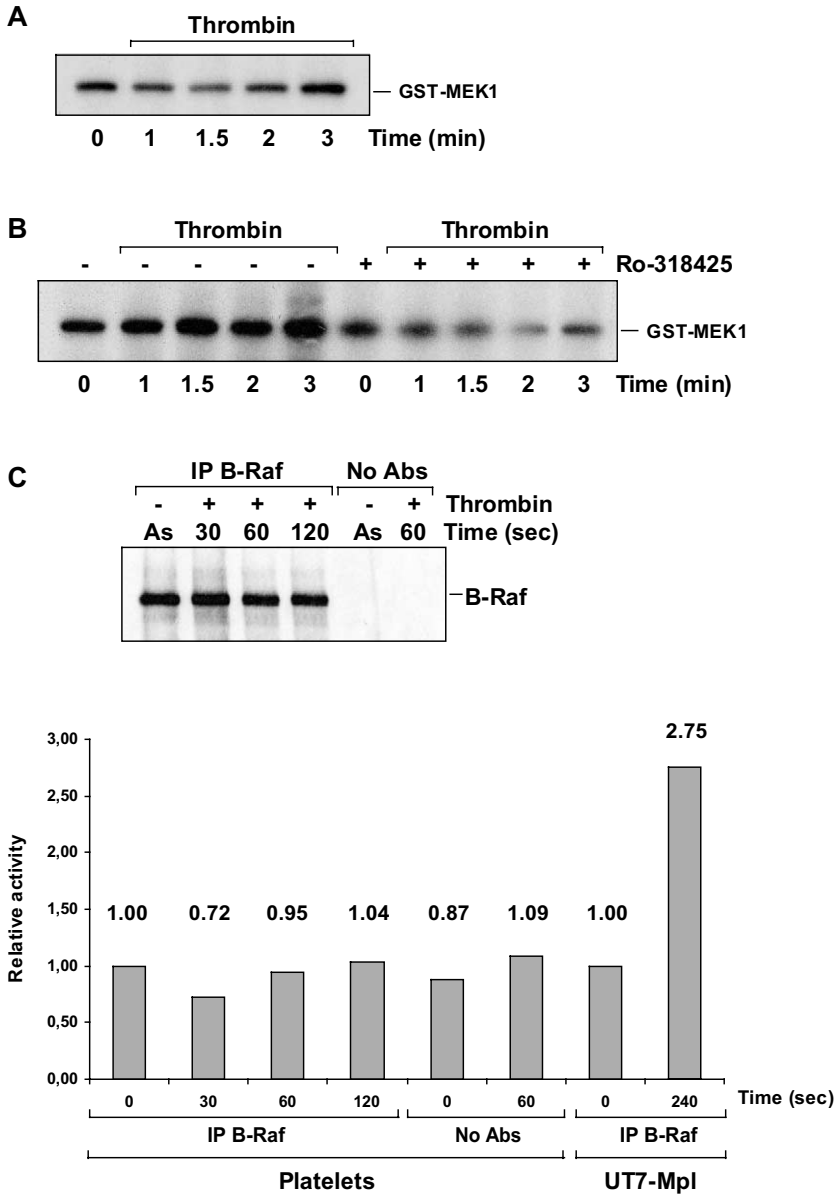

Fig. 5. B-Raf activity in thrombin-stimulated platelets. Washed platelets were preincubated in the presence of the RGDS peptide (1 mM) and then activated by thrombin (1 NIH U/ml) (A,C) for various times or (B) with 10 μM of RO318425. Samples were solubilized in Triton buffer and then immunoprecipitated with an antibody directed against B-Raf. A,B: The exogenous substrate, GST–MEK1, was phosphorylated after the immunoprecipitation of B-Raf. C: In vitro kinase assay of MEK activation: B-Raf was immunoprecipitated and then a cascade assay was carried out in which GST–MEK phosphorylated GST–ERK, which in turn phosphorylated MBP. UT7-Mpl were stimulated with 10 nM TPO mimetic peptide (4 h) as a positive control. Results are representative of three experiments.

4. Discussion

The MAPK signaling pathway induced by various agonists including thrombin [18], collagen [19] and PMA [21] is largely uncharacterized. Recently, MEK1/2 [20] and PKC [21] have been shown to be involved in ERK2 activation in human platelets, but the link between receptor and kinases remains to be established. In this study, we investigated thrombin-induced ERK2 activation and the putative kinases involved in this pathway.

We found that the inhibition of MEK1/2 by PD98059, inhibited the phosphorylation of both MEK1/2 and ERK2, consistent with previous results [20] showing that MEK1/2 is directly involved in ERK2 activation. We next investigated the possible involvement of a family of serine/threonine kinases, the PKCs. We showed, using various approaches, that calcium and conventional PKCs were involved. ERK2 activation was totally inhibited by a conventional PKC inhibitor, and calcium was necessary for the activation of both ERK2 and conventional PKCs, excluding the involvement of the new class of calcium-insensitive PKCs. PI3-kinase, which is necessary for the activation of atypical PKCs, had no effect on ERK2 activation, suggesting that PI3-kinase activation was not necessary for ERK2 activation and that atypical PKCs were not involved. Moreover, PMA, which directly activates PKCs, induced ERK2 activation, but to a lesser extent than thrombin. It is possible that the level of calcium mobilization necessary for ERK2 activation was lower after PMA induction than after thrombin induction. It is also possible that the conventional PKCs induced by thrombin and PMA are different or that the PKCs involved in ERK2 activation are necessary but not sufficient for maximal ERK2 activation. Finally, PMA may induce a specific phosphatase that has a direct effect on ERK2 activation. Our studies have shown that both calcium mobilization and conventional PKC activation are essential for ERK2 activation. Conventional PKCs were required for MEK1/2 activation, but it was unclear whether the mechanism was direct or indirect. We have investigated some possible intermediate components in ERK2 activation. MEK1/2 is activated by the serine/threonine kinase Raf-1 in proliferative cells [30]. The role of Raf-1 activation in platelets, in which it has been detected [23] is unclear. TPO may induce Raf-1 activation in platelets without subsequently inducing the activation of MEK1/2 or ERK2 [23]. In our conditions, no activation of Raf-1 was observed after thrombin stimulation. Basal phosphorylated GST–MEK1 was still observed after thrombin induction when Raf-1 was not immunoprecipitated, suggesting that the phosphorylation of GST–MEK1 is independent of Raf-1 activity and that kinases other than Raf-1 induce MEK phosphorylation on sites that are not involved in its activation. To strengthen our results, MEK activation was measured after immunoprecipitation of Raf-1. In these conditions, basal phosphorylation of MBP was observed in resting platelets and after thrombin induction in the presence or absence of the anti-Raf-1 antibody. These results confirm that MEK is not active in all conditions and suggest that basal Raf-1-independent GST–MEK phosphorylation occurs on sites that are not involved in MEK activation. Moreover, GST–MEK1 phosphorylation was still observed in the presence of conventional PKC inhibitors in resting platelets and after thrombin stimulation even though thrombin-induced ERK2 phosphorylation was totally inhibited. Finally, Raf-1 did not coimmunoprecipitate with MEK1/2 (data not shown). Taken together, our results strongly suggest that Raf-1 activity is not required for ERK2 activation.

The other isoform of Raf, B-Raf, was detected in platelets. The basal GST–MEK phosphorylation and activity detected in resting platelets and after thrombin stimulation after the immunoprecipitation of B-Raf, were still observed when B-Raf was not immunoprecipitated. Thus, B-Raf was not required for ERK2 activation and, like Raf-1, MEK phosphorylation occurs at sites that are not involved in MEK activation, probably by contaminating kinases in the precipitate.

The third isoform of Raf, A-Raf, has not been found in platelets. In proliferative cells, A-Raf is weakly activated by oncogenic ras and in proliferative cells it is strongly activated by src [34]. In platelets, we confirmed the previous finding [35] that src is not involved in thrombin-induced ERK activation (results not shown) using a src kinase inhibitor (PP1). The activation of Ras is not coupled to ERK activation in human platelets [35]. These results suggest that A-Raf is not involved in platelet ERK2 activation. Taken together, our results strongly suggest that thrombin-induced ERK activation does not involve the classical Raf-MEK1/2-ERK cascade but instead depends on calcium and the conventional PKCs–MEK1/2–ERK pathway.

Finally, phospholipase Cβ linked to Gq protein induced the formation of IP3 and diacylglycerol necessary for calcium release and PKC activation in platelets, which suggests that phospholipase Cβ is involved in upstream ERK pathway.

Our observations are consistent with recent data showing that both calcium and PKC activity contribute to the P2Y-induced activation of ERK independently of Raf-1 [36]. Moreover, activation of Ras and p42–p44 MAPKs by thrombin has been described to be uncoupled in platelets [35] suggesting that classical cascade Ras–Raf–MEK–ERK does not occur. Finally, in PC12 cells, calcium influx is necessary for ERK activation independently of A-Raf, B-Raf, and C-Raf activities [37]. Our data suggest that a MEK kinase other than Raf-1 may be activated by conventional PKCs. Alternatively, PKCs may act directly on MEK1/2. PKCs have been reported to activate MEK1/2 in COS cells both in vitro and in vivo [26]. PKCζ may act independently of Raf activation to trigger MEK and $p42_{MAPK}$ [38]. PKCζ is activated by the products of PI3-kinase and the inhibition of PI3-kinase has no effect on ERK2 activation, suggesting that this class of PKCs is not involved in MEK1/2 activation.

In conclusion, we report that thrombin-induced activation of ERK2 in human platelets is not dependent on Raf-1 or B-Raf activation but instead dependent on calcium and the conventional PKCs pathway.

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