Costimulation of the Gi-coupled ADP receptor and the Gq-coupled TXA_2 receptor is required for ERK2 activation in collagen-induced platelet aggregation

Séverine Roger[a], Marc Pawlowski[a], Aïda Habib[a,1], Martine Jandrot-Perrus[b], Jean-Philippe Rosa[a], Marijke Bryckaert[a,*]

[a] *U348 INSERM, IFR 6 Circulation Lariboisière, Hôpital Lariboisière, 41 Bd de la Chapelle, 75475 Paris Cedex 10, France*
[b] *E348 INSERM, Faculté Xavier Bichat, Paris, France*

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Abstract The stimulation of platelets by low doses of collagen induces extracellular signal-regulated kinase 2 (ERK2) activation. In this report, we demonstrate that collagen-induced ERK2 activation depends on thromboxane A_2 (TXA_2) formation and ADP release. The collagen-induced ERK2 activation was inhibited by indomethacin (88%) and by AR-C69931MX (70%), a specific antagonist of P2Y12, a Gi-coupled ADP receptor. AR-C69931MX (10 μM) inhibition was overcome by epinephrine (1 μM), an agonist of the Gi-coupled α_{2A}-adrenergic receptor, suggesting that the Gi-coupled receptor was necessary for ERK2 activation by collagen. By contrast, MRS 2179 (10 μM), a specific antagonist of P2Y1, a Gq-coupled ADP receptor, did not affect collagen-induced ERK2 activation. Little or no ERK2 activation was observed with ADP alone (10 μM). By contrast, U46619 (10 μM), a stable analog of TXA_2, induced ERK2 activation in an ADP-dependent manner, via the P2Y12 receptor. These results suggest that the Gi-dependent signaling pathway, stimulated by ADP or epinephrine, was not the only pathway required for ERK2 activation by collagen. Costimulation of the specific $G_{12/13}$-coupled TXA_2 receptor with a low dose of U46619 (10 nM) and of Gi- and Gq-coupled ADP receptor (10 μM) induced very low levels of ERK2 activation, similar to those observed with ADP alone, suggesting that $G_{12/13}$ is not involved or not sufficient to induce the additional pathway necessary for ERK2 activation. The Gq-coupled TXA_2 receptor was required for ERK2 activation by U46619 (10 μM) and low doses of collagen, clearly showing that a coordinated pathway through both Gq from TXA_2 and Gi from ADP was necessary for ERK2 activation. Finally, we demonstrate that ERK2 activation is involved in collagen-induced aggregation and secretion.

Key words: Platelet; Aggregation; Extracellular signal-regulated kinase; Collagen; P2Y12 receptor

1. Introduction

Collagen is the most thrombogenic component of the subendothelial layer following vascular injury. Collagen supports platelet adhesion to the subendothelium and subsequently induces aggregation, secretion and procoagulant activity. The interaction between collagen and platelets is complex and mediated by several receptors, including integrin α2β1, GPIV, GPVI, p65 and a TIIICBP type III collagen receptor [1,2]. The respective roles of these receptors have not been fully defined. α2β1 and GPVI are now thought to play a key role in adhesion and activation. α2β1 deficiency impairs collagen-induced platelet adhesion [3]. However, it has been demonstrated that α2β1 must be activated to acquire a high affinity for collagen [4]. Signals coupled to GPVI are proposed to be involved in α2β1 activation. Thus, GPVI appears to play a central role in activation and in the regulation of adhesion [5]. GPVI is associated with the γ chain of Fc receptors. GPVI activation by collagen induces FcRγ phosphorylation and the recruitment of the tyrosine kinase syk, followed by a cascade of signaling events, including the activation of PI3-kinase and PLCγ2 [6–8]. Subsequent events include the hydrolysis of phosphatidylinositol-4,5 bisphosphate, and the production of diacylglycerol and inositol-1,4,5 trisphosphate, which increases the cytosolic calcium concentration and activates protein kinase C (PKC) [9,10]. Autocrine agonists, such as adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2), have been reported to be involved in platelet secretion and aggregation induced by low concentrations of collagen [11]. ADP triggers changes in the affinity of α2β1 for collagen [12]. The effects of ADP on platelets are mediated by two purinergic receptors coupled to G proteins. The P2Y1 receptor coupled to Gq initiates platelet shape change by mobilizing calcium (Ca^{2+}) [13]. The recently cloned Gi-coupled P2Y12 receptor, which mediates adenylyl cyclase inhibition, is essential for full aggregation. The other mediator, TXA_2, signals through $G_{12/13}$ and Gq-coupled receptors [14]. The $G_{12/13}$-signaling pathway induces platelet shape change, involving Rho/Rho kinase-mediated phosphorylation of the myosin light chain [15] whereas Gq-mediated phospholipase Cβ activation appears to play a central role in platelet aggregation and secretion.

Collagen induces mitogen-activated protein kinase (MAPK) activation [16]. Platelet MAPKs include the extracellular signal-regulated kinase (ERKs), the c-Jun N-terminal kinase

*Corresponding author. Fax: (33)-1-49 95 85 79.
E-mail address: marijke.bryckaert@larib.inserm.fr (M. Bryckaert).

[1] Present address: American University of Beirut, Departments of Biochemistry and Internal Medicine, PO Box 11-236, Beirut, Lebanon.

Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase

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(JNK) and p38 MAPK [17–19]. ERKs are activated by various agonists, including thrombin, collagen, and TXA_2 [17]. The induction of ERK activation by thrombin is dependent on PKC and the tyrosine/threonine kinase MEK1/2, which phosphorylates ERK1/2 directly [20] but independently of the serine/threonine kinases Raf-1 and B-Raf [21]. We have shown that, in conditions of thrombin-induced platelet aggregation, ERK2 is downregulated by the engagement of αIIbβ3 integrin and is selectively dephosphorylated at the threonine^{183} position, via an unidentified serine/threonine phosphatase [22]. However, the role of ERK2 in platelets is beginning to be unraveled. The ERK cascade regulates store-mediated Ca^{2+} entry in platelets [23]. GPIb-IX mediates the activation of integrin αIIbβ3 via a MAPK pathway [24,25]. In transgenic mice overexpressing the human P2X1 ion channel, preinjection of the inhibitor of ERK2 activation protects against thrombosis [26].

In this study, we investigated the roles of ADP and TXA_2 in collagen-induced ERK2 activation. We showed that ERK2 activation induced by low doses of collagen (10 μg/ml) is dependent on the P2Y12 receptor, via ADP and TXA_2 synthesis. Moreover, whereas the analog of TXA_2, U46619, induced ERK2 activation via ADP, ADP alone failed to induce ERK2 activation, suggesting that a concomitant pathway connecting ADP and TXA_2 is necessary. We provide evidence that $G_{12/13}$ coupled to the TXA_2 receptor plays a minor role in ERK2 activation induced by collagen whereas Gq coupled to the TXA_2 receptor are involved in this concomitant pathway. Overall, our data show that the ERK2 activation required for collagen-induced aggregation and secretion is dependent on collagen receptors (GPVI and α2β1), the Gi-coupled P2Y12 receptor and the Gq-coupled TXA_2 receptor.

2. Materials and methods

2.1. Reagents

Type I collagen from fetal bovine skin was obtained from Chemicon International (Temecula, CA, USA). Convulxin (Cvx) was generously provided by Dr. M. Jandrot Perrus (Paris, France). The synthetic peptide Arg-Gly-Asp-Ser (RGDS), (α,β-methylene) triphosphate 5′-triphosphate (α,β-MeATP), leupeptin, aprotinin, dimethylsulfoxide, apyrase and epinephrine were purchased from Sigma (St. Louis, MO, USA). Indomethacin and SQ29548 (5-heptenoic acid, 7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2-2-1]-hept-2-yl]-,[1S-[1α,2α(Z), 3α, 4α]) were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). U46619 (9,11-dideoxy-9α,11α-methanoepoxyprostaglandin F2α), Y-27632 and stearic acid-MPKKKPTPIQLNP (Ste-MKPPPPTPIQLNP) were obtained from Calbiochem (Meudon, France). 5-Hydroxy[side-chain-2-^{14}C]tryptamine creatine sulfate (1.85–2.29 GBq mmol) was obtained from Amersham (Buckinghamshire, England). AR-C69931MX and MRS 2179 were generously provided by Dr. B. Humphries (Astra Zeneca, UK) and Dr. C. Gachet (INSERM 311, Strasbourg, France). Fibrinogen was obtained from Kordia (Leiden, The Netherlands). The anti-α2β1 integrin monoclonal antibody 6F1 was generously provided by Dr. Barry S. Coller (New York, USA). The anti-GPVI monoclonal antibody 9O12.2 Fab fragments were generously provided by Dr. C. Lecut (Paris, France). Mouse monoclonal antibody directed against the phosphorylated form of ERK_S (ERK_S-P) was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Polyclonal antibody directed against the phosphorylated form of p38 MAPK was purchased from Promega (Madison, WI, USA). Peroxidase-conjugated affinity-pure donkey anti-rabbit IgG was obtained from Jackson Immunoresearch Laboratories (West Grove, DA, USA).

2.2. Isolation of platelets

Venous blood was collected from healthy donors free of medication for at least two weeks prior to blood collection. Conventional informed consent was obtained from all donors, in accordance with the guidelines of the committee of the French Blood Transfusion Agency (*l'Etablissement Française de Transfusion Sanguine*). Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at $120\times g$ for 15 min at 20°C, and platelets were isolated by differential centrifugation once, in citrate buffer, pH 6, supplemented with 10^{-4} mM prostaglandin E1, 0.1 U/ml of the ADP scavenger apyrase (adenosine-5′-triphosphate diphosphohydrolase), 140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM saccharose and then again in the same buffer but without prostaglandin E1 and apyrase. The platelet pellet was resuspended in 10 mM HEPES, pH 7.4, 140 mM NaCl, 3 mM KCl, 5 mM $NaHCO_3$, 0.5 mM MgCl and 10 mM glucose. Cell concentration was adjusted to 5×10^8/ml.

2.3. Platelet aggregation

Platelet aggregation (0.4 ml samples) was assessed in a Chronolog dual-beam aggregometer with constant stirring (1200 rpm) at 37°C. Fibrinogen (0.25 mg/ml) was added to samples in the aggregometer prior to the addition of methyl-thio-ADP (MeSADP, 10 μM). The platelets were incubated with various inhibitors, without stirring, at 37°C, for various times before adding agonists (collagen (10–15 μg/ml), MeSADP (10 μM), U46619 (10 nM–10 μM), epinephrine (1 μM), Cvx 1 nM). Aggregation was measured and expressed as a percent change in light transmission, with the value for the blank sample (buffer without platelets) set at 100%.

2.4. Platelet secretion

PRP was incubated with [^{14}C]5-HT (0.5 μCi/10 ml PRP) for 30 min at 37°C. Platelets were then isolated as described below. [^{14}C]5-HT secretion was measured in conditions of platelet aggregation. After 2 min of incubation, we added ice-cold EDTA. The samples were centrifuged for 3 min and the supernatant was removed for liquid scintillation counting.

2.5. Thromboxane B_2 (TXB_2) measurement

Washed human platelets (400 μl, 5×10^8/ml) were stimulated by incubation in an aggregometer with or without collagen (10 μg/ml) in the presence or absence of other reagents (AR-C69931MX (10 μM), MRS 2179 (10 μM)) at 37°C, with stirring. The reaction was stopped after 2 min of collagen activation, by quickly adding in ice-cold EDTA (10 mM). The samples were centrifuged at $3000\times g$ for 1 min. TXB_2, the stable metabolite of TXA_2, was quantified by EIA as previously described [27].

2.6. Immunoblotting

Samples were subjected to immunoblotting as previously described [18]. Briefly, platelets were lysed in sodium dodecyl sulfate (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 leupeptin, 10 μg/ml aprotinin, pH 7.4) and heated at 95°C for 5 min. Proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) in 12% or 8% acrylamide gels and transferred to nitrocellulose filters by semi-dry transfer (Enportech, Natrich, MA, USA). Filters were then incubated overnight at 4°C with the polyclonal primary antibody against ERK-P (1/10 000) and reprobed for 1 h at room temperature with antibodies against ERKs (1/20 000). The membranes were washed five times in phosphate-buffered saline without milk and incubated with peroxidase-conjugated rabbit anti-mouse (1/20 000) or peroxidase-conjugated donkey anti-rabbit (1/20 000) antibody for 45 min at room temperature. Immunoreactive bands were visualized with enhanced chemiluminescence detection reagents (Pierce).

2.7. Statistics

Results are expressed as means ± S.E.M. for at least three independent experiments. Statistical significance was assessed with Student's *t*-test for paired comparisons.

3. Results

3.1. Collagen-induced ERK2 activation requires α2β1 integrin and GpVI

3.1.1. Collagen induces ERK2 activation. We first determined the conditions required for collagen-induced ERK2

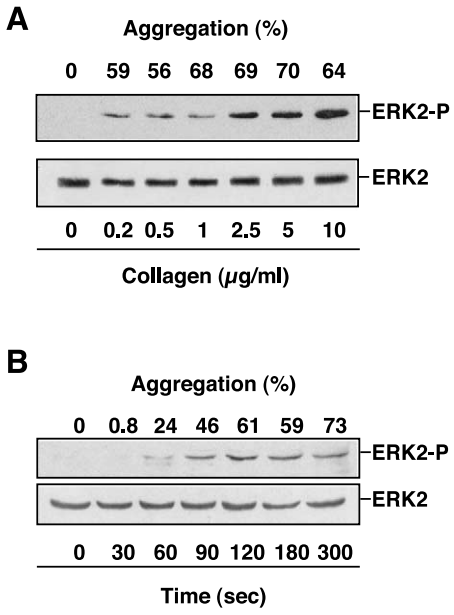

Fig. 1. Effect of collagen-induced ERK2 activation. Washed human platelets were stimulated by incubation for 2 min at 37°C, with stirring, at various concentrations of collagen (0–10 μg/ml) (A) or for various periods of time (0–300 s) with collagen (10 μg/ml) (B). ERK2 phosphorylation was analyzed by SDS–PAGE, followed by Western blotting, using a monoclonal antibody specific for ERK2-P and a polyclonal antibody recognizing total ERK2. Autoluminograms are representative of at least three independent experiments.

activation, by incubating platelets with various concentrations of collagen (0–10 μg/ml) for 2 min, with stirring. In resting platelets, ERK2 was not phosphorylated. Phosphorylated ERK2 (ERK2-P) was detected in the presence of low concentrations of collagen (0.2–1 μg/ml) and ERK phosphorylation increased significantly with increasing collagen concentration (2.5–10 μg/ml) (Fig. 1A). In parallel, we investigated the pattern of ERK2 activation over time upon treatment with 10 μg/ml collagen, with stirring. ERK2-P was not detected in resting platelets and platelets stimulated with collagen for 30 s (Fig. 1B). ERK2-P was detected after 60 s of collagen activation, when there was about 24% platelet aggregation. ERK2-P levels peaked at 120 s and then decreased. Thus, our results confirm that ERK2 is activated in a time- and dose-dependent manner by collagen.

3.1.2. α2β1 integrin and GpVI are involved in ERK2 activation. We then investigated the role of the collagen receptor α2β1 integrin by adding a function-blocking monoclonal antibody specific for α2β1 integrin (6F1) in conditions of collagen-induced ERK2 activation (Fig. 2A). The addition of 6F1 (10 μg/ml) totally inhibited collagen-induced ERK2 activation, strongly suggesting that α2β1 integrin is required for ERK2 activation. Collagen-induced ERK2 phosphorylation was not inhibited when platelet aggregation was inhibited by the RGDS peptide, suggesting that αIIbβ3 integrin does not participate in ERK2 activation (results not shown).

As Cvx is a selective GPVI activator, we next investigated Cvx-induced ERK2 activation in conditions of platelet aggregation. ERK2 was phosphorylated with 1 nM Cvx, indicating that GPVI is coupled to ERK2 activation (Fig. 2B). In these conditions, platelet aggregation and ERK2 phosphorylation were not inhibited by 6F1 (Fig. 2B), suggesting that GPVI alone is able to induce ERK2 activation. In parallel, we investigated the GPVI-dependent signaling pathway activated by collagen. Platelets, pretreated with anti-GPVI monoclonal antibody 9O12.2 Fab fragments [28], were stimulated with collagen (15 μg/ml). 9O12.2 (50 μg/ml) inhibited collagen-induced ERK2 activation, suggesting strongly that a signaling pathway induced by GPVI is involved in collagen-induced ERK2 activation (Fig. 2B). Thus, both GPVI and α2β1 integrin are required for full ERK2 activation by collagen.

3.2. ADP is necessary for collagen-induced ERK2 activation

3.2.1. ADP-triggered P2Y12 activation is required for collagen-induced ERK2 phosphorylation. As ADP activates multiple G protein pathways, including the Gq and Gi pathways for ADP, to induce platelet shape change and platelet aggregation, we investigated the role of this mediator in collagen-

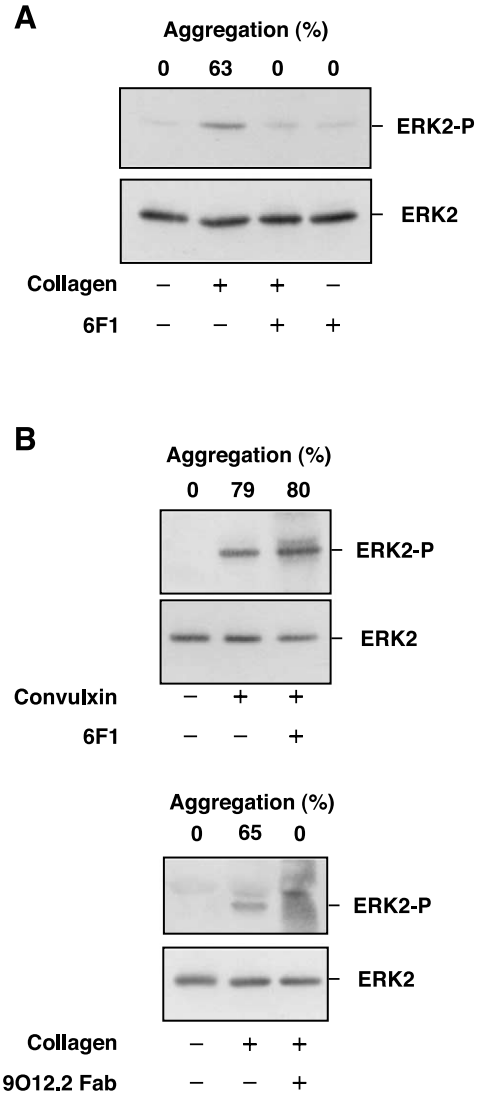

Fig. 2. Role of GPVI and α2β1 integrin in ERK2 activation. Washed human platelets were stimulated by incubation for 2 min at 37°C, with stirring, with (A,B) collagen (10 μg/ml) or (B) Cvx (1 nM) in the presence or absence of an antibody against α2β1 integrin (6F1) (10 μg/ml: A,B) or anti-GPVI monoclonal antibody 9O12.2 Fab fragments (50 μg/ml). ERK2 phosphorylation was analyzed by SDS–PAGE followed by Western blotting with a monoclonal antibody specific for ERK2-P and a polyclonal antibody recognizing total ERK2. Autoluminograms are representative of at least three independent experiments.

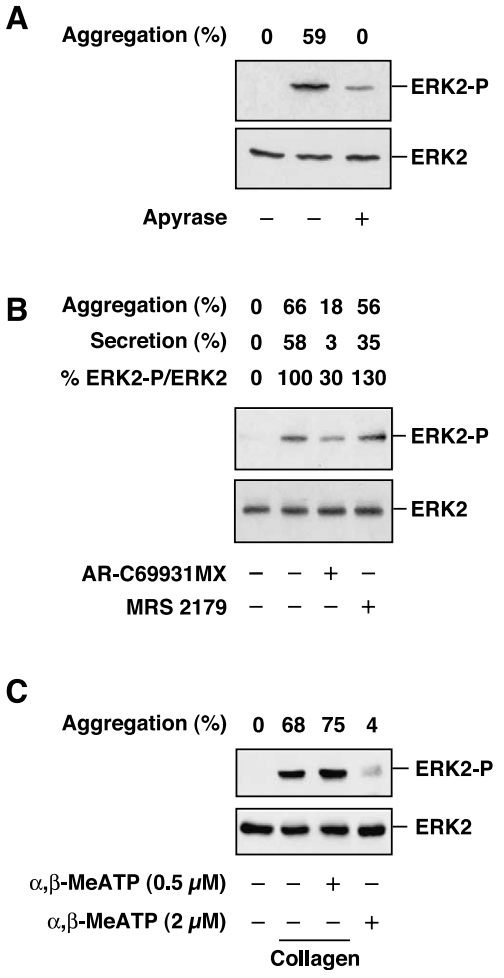

Fig. 3. Effect of ADP on collagen-induced ERK2 activation. Platelets were stimulated by incubation with collagen (10 μg/ml) for 2 min, at 37°C, with stirring, (A) in the presence or absence of apyrase (1 U/ml) or (B) AR-C69931MX (10 μM) and MRS 2179 (10 μM). C: α,β-MeATP (0.5 μM) and collagen (10 μg/ml) were simultaneously added to apyrase-untreated platelets for 2 min at 37°C, with stirring. Aggregation and secretion were measured. In parallel, ERK2 phosphorylation was analyzed by SDS–PAGE followed by Western blotting using a monoclonal antibody specific for ERK2-P and a polyclonal antibody recognizing total ERK2. Autoluminograms are representative of at least three independent experiments.

induced ERK2 activation. When platelets were preincubated with apyrase (1 U/ml), ERK2 activation induced by collagen was partially inhibited (Fig. 3A). We thus investigated the role of the P2Y12 and P2Y1 receptors in ERK2 activation. We used two selective antagonists: AR-C69931MX, which is specific for the P2Y12 receptor, and MRS 2179, which is specific for the P2Y1 receptor [29]. Strong inhibition of the aggregation (73%) and secretion (95%) induced by collagen (10 μg/ml) was observed after 2 min of incubation with collagen in the presence of AR-C69931MX (10 μM), whereas platelet aggregation and secretion were only slightly inhibited by the antagonist of the P2Y1 receptor, MRS 2179 (10 μM) (Fig. 3B). In these conditions, collagen-induced ERK2 phosphorylation (100% in the absence of inhibitor) was significantly inhibited by AR-C69931MX, reaching 30 ± 6%, whereas MRS 2179 had no significant effect (130 ± 32% ERK2 phosphorylation). Thus, ADP plays a crucial role in collagen-induced aggregation, secretion and ERK2 activation. Moreover, this activation of ERK2 is mostly dependent on the Gi-coupled P2Y12 receptor.

At a low dose of collagen (1 μg/ml), ERK2 phosphorylation has been reported to be mediated by the P2X1 receptor [30]. In our conditions (10 μg/ml collagen), the addition of α,β-MeATP (0.5 μM) had no effect on collagen-induced platelet aggregation and ERK2 activation, demonstrating that ERK2 activation did not require the P2X1 receptor at this dose of collagen (10 μg/ml) (Fig. 3C). By contrast, α,β-MeATP (2 μM) alone induced weak phosphorylation of ERK2, as previously described [29], suggesting that the P2X1 receptor was not desensitized in our conditions (Fig. 3C).

To confirm the role of the Gi pathway in collagen-induced ERK2 activation, we investigated whether epinephrine, the specific ligand of the Gi-coupled α_{2A} adrenoreceptor, could overcome the inhibition of ERK2 activation by AR-C69931MX. In these conditions, in which AR-C69931MX (10 μM) inhibited both collagen-induced aggregation and

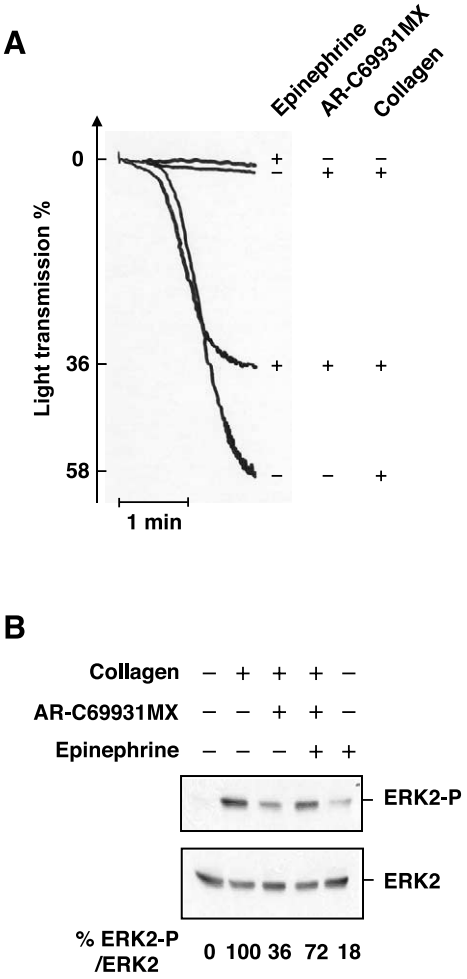

Fig. 4. Effect of epinephrine on AR-C69931MX-induced inhibition of ERK2 activation. A: Platelets were stimulated by incubation for 2 min, at 37°C, with collagen (15 μg/ml), with stirring, in the presence or absence of AR-C69931MX (10 μM), with or without epinephrine (1 μM). B: In parallel, ERK2 phosphorylation was analyzed by SDS–PAGE followed by Western blotting using a monoclonal antibody specific for ERK2-P and a polyclonal antibody recognizing total ERK2. Relative activities are indicated below as the ratio of ERK2-P over total ERK in %. Results are representative of four independent experiments.

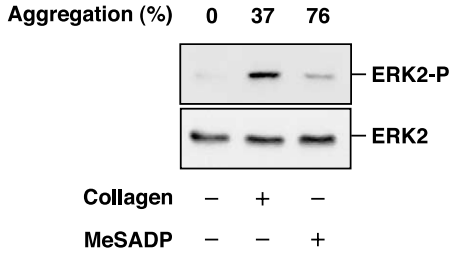

Fig. 5. Effect of MeSADP on ERK2 activation. Platelets were stimulated with collagen (10 μg/ml) or MeSADP (10 μM). ERK2 phosphorylation was analyzed by SDS–PAGE followed by Western blotting using a monoclonal antibody specific for ERK2-P and a polyclonal antibody recognizing total ERK2. Autoluminograms are representative of at least three independent experiments.

ERK2 activation, the addition of epinephrine (1 μM) partially restored aggregation (62%) and ERK2 phosphorylation (72%) (Fig. 4A,B). By contrast, epinephrine alone (1 μM), in the absence of collagen, induced only weak ERK2 activation. In conclusion, these results show that the Gi-dependent pathway is involved in collagen-induced ERK2 activation.

3.2.2. MeSADP alone is not sufficient to induce full ERK2 phosphorylation. By contrast, when platelets were stimulated with MeSADP (10 μM), little or no ERK2 phosphorylation was observed depending on the experiment (Fig. 5). In these conditions, full aggregation (76%) and Ca^{++} mobilization (results not shown) were observed, suggesting that the P2Y1 receptor of ADP was functional and not desensitized. These results indicate that MeSADP alone is not sufficient to induce full ERK2 activation and suggest that a concomitant signaling pathway, linking the Gi-coupled P2Y12 receptor for ADP and another pathway, is required for efficient collagen-induced ERK2 activation.

3.3. TXA_2 is required for ERK2 activation

As collagen is known to induce TXA_2 formation, we investigated its role, using indomethacin, an inhibitor of cyclooxygenase. At an indomethacin concentration of 5 μM, which inhibited collagen-induced aggregation (88 ± 6%) and secretion (95 ± 4%) (Fig. 6A), the level of ERK2 phosphorylation was decreased by 88%, suggesting a major role for TXA_2 in collagen-induced ERK2 activation (Fig. 6A). These results were confirmed using the antagonist of the TXA_2 receptor, SQ29548 (1 μM) (results not shown). Thus, collagen-induced ERK2 activation is dependent on the Gi-coupled P2Y12 receptor and on TXA_2 effect. In parallel, stimulation by collagen induction resulted in the synthesis of 42 ng TXB_2 ± 8 ng/10^8 platelets, the stable derivative of TXA_2 (results not shown). In the presence of AR-C69931MX, we observed weak but significant inhibition of TXB_2 synthesis (30.5 ± 3 ng/10^8 platelets; $P < 0.01$; 27.4% inhibition) whereas MRS 2179 had no effect (43 ± 4 ng/10^8 platelets). Thus, only 30% collagen-induced TXA_2 synthesis is dependent on ADP.

We investigated whether the TXA_2 receptor was directly involved in ERK2 activation. U46619 (10 μM) induced ERK2 activation (Fig. 6B), which was mostly dependent on the P2Y12 receptor because AR-C69931MX (10 μM) inhibited ERK2-P by 70%. The P2Y1 receptor was not involved because MRS 2179 had no effect. Moreover, the RGDS peptide did not affect U46619-induced ERK2 activation, suggesting that αIIbβ3 engagement is not involved in ERK2 activation (results not shown). Thus, the TXA_2-stimulated pathway leading to ERK2 phosphorylation is partially dependent on the Gi-coupled P2Y12 receptor pathway and independent of the Gq-coupled P2Y1 receptor pathway.

3.4. $G_{12/13}$ coupled to the TXA_2 receptor plays a minor role in collagen-induced ERK2 activation

TXA_2 activates multiple G protein pathways including the $G_{12/13}$ and Gq pathways. We investigated whether the $G_{12/13}$-coupled TXA_2 receptor was involved in the concomitant pathway necessary for ERK2 activation.

We first determined the conditions in which U46619 induced only the $G_{12/13}$ signaling pathway, without stimulating the Gq pathway. Low concentrations (10 nM) of U46619 induced a shape change as previously described [31], consistent with the activation of $G_{12/13}$ alone (Fig. 7A). In the

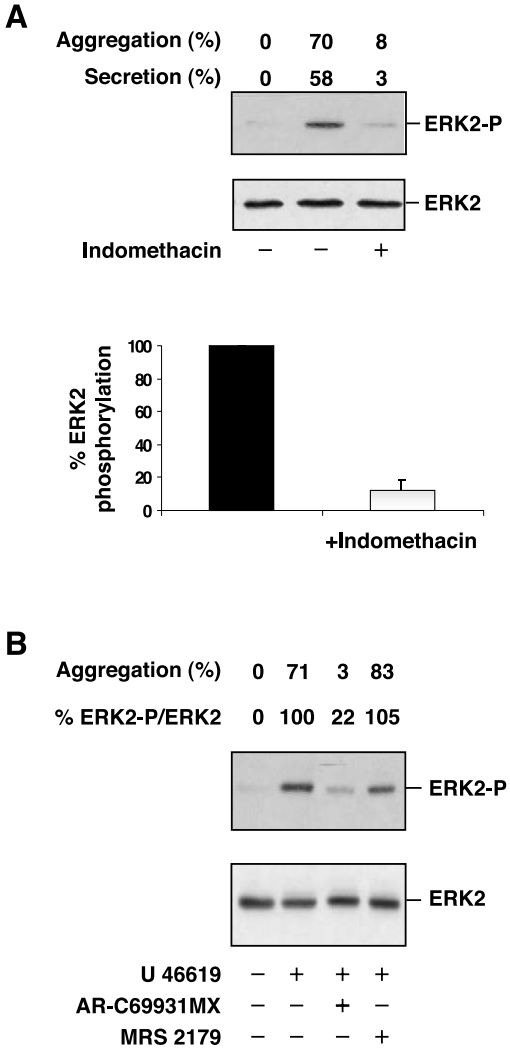

Fig. 6. Effect of TXA_2 on collagen-induced ERK2 activation. Platelets were stimulated (A) with collagen (15 μg/ml) for 2 min, with stirring, in the presence or absence of indomethacin (5 μM) or (B) with U46619 (10 μM) for 2 min, at 37°C, with stirring, in the presence or absence of AR-C69931MX (10 μM) and MRS 2179 (10 μM). Aggregation and secretion were measured. In parallel, ERK2 phosphorylation was analyzed by SDS–PAGE followed by Western blotting using a monoclonal antibody specific for ERK2-P and a polyclonal antibody recognizing total ERK2. The quantitative ERK2-P results obtained by densitometric analysis are representative of at least three independent experiments.

presence of U46619 (10 nM), very weak activation of ERK2 was observed, showing that the $G_{12/13}$-mediated signaling pathway alone is not sufficient to induce full ERK2 activation. We stimulated platelets with MeSADP (10 μM), U46619 (10 nM) or both. Basal levels of ERK2 activation induced by U46619 were not significantly increased by MeSADP (Fig. 7B). Our results suggest that the combination of $G_{12/13}$ coupled to the TXA_2 receptor and Gi and Gq coupled to ADP receptors is not sufficient to induce full ERK2 activation.

3.5. Gq coupled to the TXA_2 receptor is involved in collagen-induced ERK2 activation

The main signaling pathway dependent on the Gq-coupled TXA_2 receptor involves the activation of PKCs. We previously showed that ERK2 activation induced by thrombin is dependent on conventional PKCs [21]. We investigated the role of PKCs dependent on the Gq-coupled TXA_2 receptor in ERK2 activation. Platelets were incubated with GF109203X (10 μM), which inhibits PKCs, before activation with collagen (10 μg/ml) or U46619 (10 μM). GF109203X inhibited collagen- and U46619-induced ERK2 activation (Fig. 8), demonstrating that PKCs are involved in ERK2 activation. Thus, the Gq-coupled TXA_2 receptor and the Gi-coupled ADP receptor seem to be required for ERK2 activation.

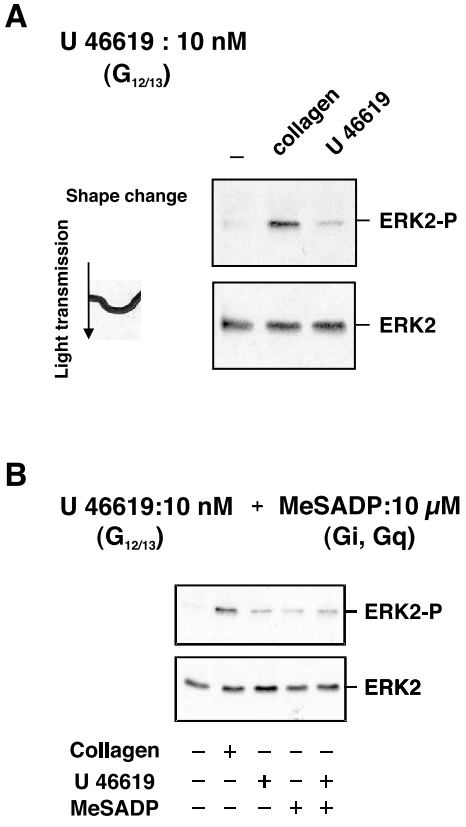

Fig. 7. Involvement of the $G_{12/13}$ signaling pathway in ERK2 activation. A: Platelets were stimulated by incubation with U46619 (10 nM) for 2 min, at 37°C, with stirring. Platelet shape change was assessed in a Chronolog dual beam aggregometer with constant stirring (1200 rpm) at 37°C and ERK2 phosphorylation was quantified and analyzed. B: The ERK2 phosphorylation induced by U46619 (10 nM) ($G_{12/13}$) with or without MeSADP (10 μM) (Gi, Gq) was analyzed. Results are representative of at least four independent experiments.

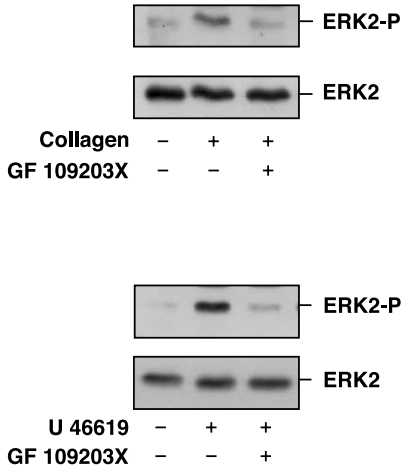

Fig. 8. Role of PKCs in collagen-induced ERK2 activation. Collagen- and U46619-induced ERK2 phosphorylation were analyzed in the presence or absence of GF109203X (10 μM). Results are representative of at least three independent experiments.

3.6. ERK2 activation is required for collagen-induced aggregation and secretion

Finally, we investigated the role of ERK2 in collagen-induced platelet aggregation and secretion, using a specific peptide derived from the amino terminus of MEK1 that bound ERK2 [32]. Platelets were first incubated for 30 min with various concentrations of the non-toxic permeable peptide Ste-MPKKKPTPIQLNP (2–10 μM), a competitive inhibitor of the MEK1/2, before addition of collagen (15 μg/ml). In these conditions, Ste-MPKKKPTPIQLNP inhibited platelet aggregation dose dependently up to 81% at a concentration of 10 μM (Fig. 9A). In parallel, we evaluated the phosphorylation of ERK2 and p38 MAPK. Ste-MPKKKPTPIQLNP (2–10 μM) did not affect collagen-induced p38 phosphorylation, regardless of the concentration used. By contrast, ERK2 phosphorylation was inhibited by 4 μM of Ste-peptide, a concentration that inhibited platelet aggregation (Fig. 9B). Finally, we assessed the effect of Ste-MPKKKPTPIQLNP on collagen-induced platelet secretion. Total inhibition of secretion (100%) was observed only at a concentration of peptide (5 μM) that inhibited platelet aggregation by 71% (Fig. 9C). By contrast, 2 μM of a control peptide, which did not inhibit ERK2 phosphorylation, had no effect on platelet aggregation and secretion (112 ± 15% and 98 ± 21%, respectively, with the values for collagen alone taken as 100%). Thus our results strongly suggest that ERK2 activation is involved in collagen-induced platelet aggregation and secretion.

4. Discussion

MAPKs are ubiquitously expressed and their signaling cascade has been shown to participate in many cellular processes, including differentiation, adhesion, migration, proliferation and death. Platelets, which express various families of MAPKs including ERK2, JNK1 and p38 MAPKs, are activated by stimuli like thrombin and collagen [16–18]. We recently found that, in contrast to what has been observed in proliferative cells, the ERK2 cascade induced by thrombin is

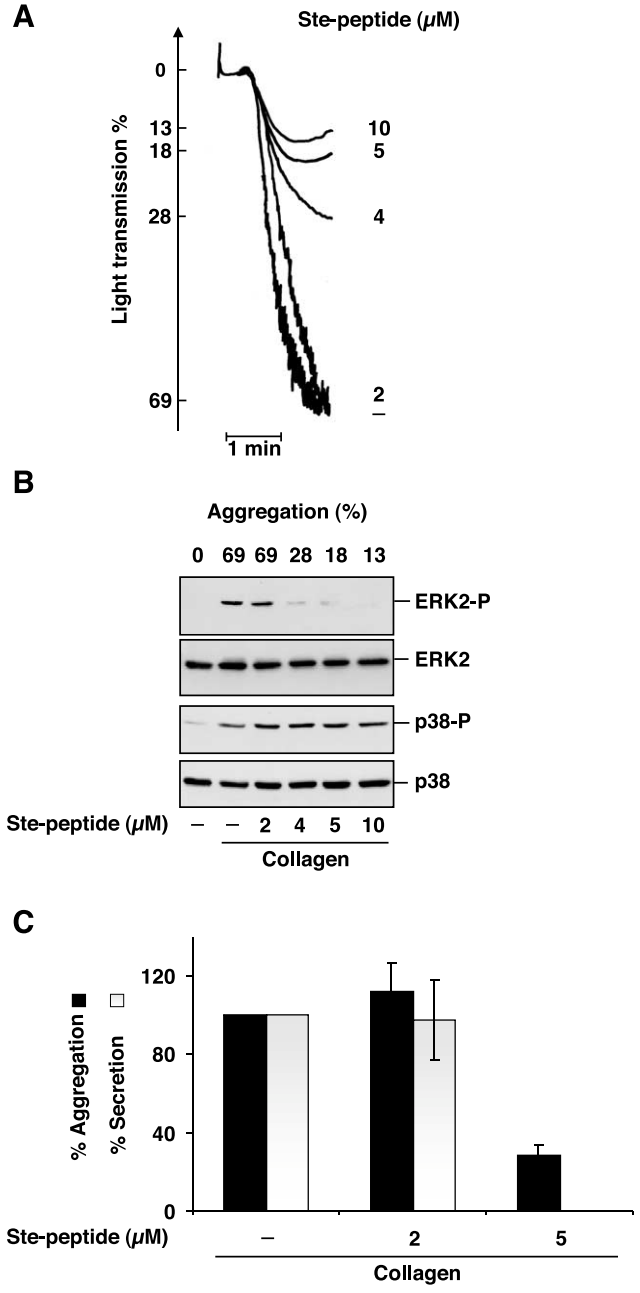

Fig. 9. Involvement of ERK2 activation in collagen-induced platelet aggregation and secretion. Platelets were first treated with Ste-MPKKKPTPIQLNP (2–10 μM) for 30 min and then stimulated with collagen (15 μg/ml). A: Aggregation was measured. B: ERK2 and p38 phosphorylation were analyzed by SDS–PAGE, followed by Western blotting using a monoclonal antibody specific for ERK2-P and a polyclonal antibody recognizing total ERK2, p38-P and total p38. Autoluminograms are representative of at least three independent experiments. C: Secretion and aggregation were normalized and expressed as a percent with respect to collagen alone (taken as 100%) ± S.E.M.

independent of the serine/threonine kinases Raf-1 and B-Raf, but dependent on conventional PKCs and calcium [21]. In this study, we addressed the question of the triggering of the ERK2 signaling pathway in conditions of platelet aggregation induced by low doses of collagen. We demonstrated that collagen-dependent ERK2 activation involved two autocrine mediators, ADP and TXA_2. This activation was dependent on the Gi-coupled P2Y12 receptor of ADP and was independent of the Gq-coupled P2Y1 receptor. Moreover, the P2Y12 receptor for ADP was required for the activation of ERK2 induced by the stable analog of TXA_2, U46619. Surprisingly, ADP alone, in conditions of platelet aggregation, was not sufficient to induce full ERK2 activation. This provides strong evidence that, in conditions of collagen activation, ERK2 phosphorylation requires the Gi-coupled P2Y12 receptor of ADP and an additional pathway working in combination with ADP. The $G_{12/13}$-coupled TXA_2 receptor played a minor role in collagen-induced ERK2 activation, and is therefore unlikely to be involved in the concomitant pathway. In contrast, the Gq-coupled TXA_2 receptor was essential for collagen-induced ERK2 activation. Our data show that a coordinated pathway between the Gi-coupled P2Y12 receptor for ADP and Gq-coupled TXA_2 is required for collagen-induced ERK2 activation, and therefore for platelet aggregation and secretion.

Platelets interact with collagen through a variety of receptors, including α2β1 integrin, GPVI, GPIV, and 65 and 85 kDa proteins [1,2]. Two major surface receptors, the integrin α2β1 and GPVI are involved in this interaction. Cvx, a selective activator of GPVI [33], induced ERK2 activation. Moreover, both the Fab fragments of an antibody directed against GPVI and antibody 6F1 directed against α2β1 integrin inhibited collagen-induced ERK2 activation, which is consistent

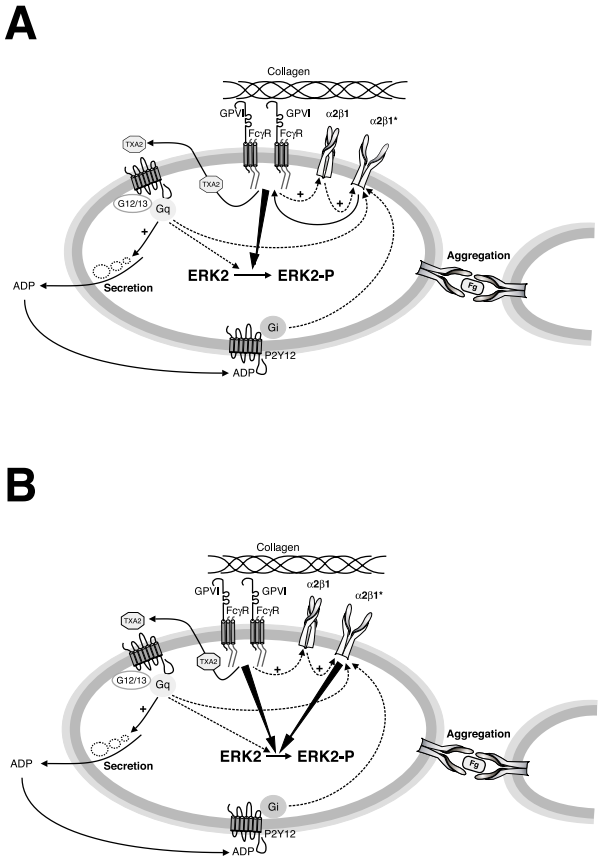

Fig. 10. Model of collagen-induced ERK2 activation. Platelets stimulated with collagen via GPVI and α2β1 integrin release ADP and produce TXA_2. Both ADP and TXA_2 induce α2β1 activation. A: α2β1 integrin activation stabilizes GPVI interactions, enhancing GPVI signaling like ERK2 activation. B: Alternatively, ERK2 activation may occur downstream of α2β1 integrin as a consequence of GPVI signaling.

with a role for α2β1 integrin and GPVI. The link between GPVI and α2β1 integrin is still unclear. In mice with β1-null platelets, GPVI, but not α2β1 integrin, has been reported to be essential for platelet interaction and activation with collagen [34]. In contrast, in ex vivo thrombus formation during the perfusion of whole blood over collagen, these receptors – GPVI and α2β1 – have complementary functions [35]. α2β1 has been reported to be activated by inside-out signals from a G-coupled receptor of ADP, with the P2Y12 receptor playing a predominant role [12]. Moreover, crosstalk between GPVI and Gi-coupled receptors during collagen-induced platelet aggregation has been reported [36]. In our model, α2β1, GPVI and the P2Y12 receptor are required for collagen-induced ERK2 activation. We propose that GPVI is the primary and the central collagen receptor that activates α2β1 integrin as an inducible high-affinity receptor and induces the release of ADP and TXA_2 formation (both also activate integrin). Consequently, activation of α2β1 stabilizes GPVI interactions, which enhances GPVI signaling like ERK2 activation (Fig. 10). We cannot exclude the possibility that ERK2 activation occurs downstream of α2β1 integrin as a consequence of GPVI signaling.

How does the Gi-coupled ADP receptor function in the activation of ERK2? Gi is known to inhibit adenylate cyclase and to reduce cAMP levels [37]. PI3 kinase, a candidate signaling intermediate acting via Gi-coupled P2Y12, does not seem to be involved in collagen-induced ERK2 activation because selective inhibitors of PI3 kinase did not significantly affect collagen-induced ERK2 activation (results not shown). Activation of the small GTPase Rap1B in human platelets was recently reported to require a Gi-dependent pathway [38,39]. Moreover, the activation of Rap1 mediates sustained MAPK activation via B-Raf activation in proliferative cells [40]. We have shown that B-Raf is not activated in conditions of thrombin induction [21], suggesting that B-Raf is not involved in ERK2 activation. However, we cannot exclude the possibility that collagen-induced ERK2 activation involves B-Raf activation.

Consistent with the results of a previous study [30], we observed little or no ERK2 activation with ADP alone, which resulted in calcium mobilization (results not shown) and full aggregation, conditions in which the P2Y1 receptor is functional. Our results suggested that an additional pathway, in combination with that involving the Gi-coupled P2Y12 receptor, was necessary for collagen-induced ERK2 activation. Conversely, the analog of TXA_2, U46619, which induced ERK2 activation, was partly dependent on the P2Y12 receptor. A pathway dependent on $G_{12/13}$ and/or the Gq-coupled TXA_2 receptor may be required. Little or no ERK2 activation was observed in conditions in which only the $G_{12/13}$-coupled TXA_2 receptor (U46619: 10 nM) was coactivated with Gi- and Gq-coupled ADP receptors (10 μM), suggesting that $G_{12/13}$ was not required or not sufficient to induce full ERK2 activation with ADP. The other G protein, the Gq-coupled TXA_2 receptor, which induces PKC activation, is involved in platelet secretion [14]. We cannot rule out the possible involvement of the P2X1 receptor, which has been reported to induce ERK2 activation in the presence of low concentrations of collagen (1 μg/ml) [30]. In our conditions (10 μg/ml collagen), desensitization of the P2X1 receptor by α,β-MeATP did not affect ERK2 activation, suggesting that higher doses of collagen required the P2Y12 receptor rather than the P2X1 receptor. Moreover, in this previous study, in contrast to our study, ERK2 activation was not affected by TXA_2 synthesis and ADP release. Finally, the abolition by PKC inhibition of the ERK2 activation induced by collagen and U46619 strongly suggests that the Gq-coupled TXA_2 receptor is a good candidate for the concomitant pathway. In conditions of collagen induction, a role of PKCs via the activation of GPVI cannot be completely excluded. Thus, ERK2 activation appears to require the Gq signaling pathway induced by TXA_2, involved in the release of mediators such as ADP, acting through Gi-coupled receptors. Finally, the fact that the Gq-coupled P2Y1 receptor is not able to induce ERK2 activation is probably due to the level of the P2Y1 receptor (250 copies) being too low to induce the strong signal required for ERK2 activation. Alternatively, the signaling pathways of the Gq-coupled P2Y1 receptor and Gq-coupled TXA_2 receptor may be different.

The role of ERK2 in collagen-induced secretion and aggregation is still unclear. Collagen-induced dense granule release was decreased by a P2Y12 receptor antagonist. The Gi pathway is essential for the amplification of secretion and aggregation and the participation of ERK2 in these events cannot be excluded. In cases in which ERK2 activation is inhibited, a decrease in platelet aggregation and secretion induced by low doses of agonists such as collagen, von Willebrand factor and thrombin has been reported [24,41]. In our conditions, PD98059, the inhibitor of the MAPK pathway, strongly inhibited TXA_2 synthesis (by 80%; results not shown) but not arachidonic acid release (results not shown). Our results confirm that PD98059 acts on cyclooxygenase activity, as reported previously [42]. Consistent with inhibition of cyclooxygenase dependent-TXA_2 production by PD98059, indomethacin inhibited collagen-induced ERK2 activation. Another strategy was used, with a stearated form of the MEK1-derived peptide inhibitor 1 (Ste-MPKKKPTPIQLNP) [32]. In these conditions, in which ERK2 was inhibited but p38 was not, collagen-induced platelet aggregation and secretion were inhibited, providing evidence that ERK signaling was involved in platelet function.

In conclusion, this study reports that a costimulation pathway involving the Gi-coupled P2Y12 receptor for ADP and the Gq-coupled TXA_2 receptor is required for the induction of ERK2 activation necessary for platelet secretion and aggregation. Our data show that collagen-induced ERK2 activation is a complex integrated process involving various specific G proteins required for full platelet activation.

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