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IN FOCUS

NF-κB inhibitors impair platelet activation responses

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Summary. *Background:* Although platelets are anucleated cells, they express several transcription factors that exert non-genomic functions, including the positive and negative regulation of platelet activation. NF-κB is a major transcriptional regulator of genes involved in survival, proliferation and inflammation. *Objective:* Because platelets play a critical role not only in hemostasis, but also in inflammation and tumor progression, we evaluated the role of NF-κB in platelet physiology. *Results:* Immunofluorescence, Western blotting and ELISA studies revealed that platelets express IκBα and NF-κB, and that stimulation with thrombin triggers IκBα phosphorylation and degradation and the binding of platelet NF-κB p65 subunit to synthetic olignoucleotides containing the consensus sequence for NF-κB. Two specific unrelated inhibitors of NF-κB activation, BAY 11-7082 and Ro 106-9920, reduced PAC-1 and fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ and restricted platelet spreading on immobilized fibrinogen. Both inhibitors impaired aggregation mediated by ADP, epinephrine, collagen or thrombin, but not arachidonic acid. ATP release, TXB_2 formation, P-selectin expression, ERK phosphorylation and $cPLA_2$ activity stimulated by thrombin were reduced in BAY 11-7082- or Ro 106-9920-treated platelets. Although bleeding time was not affected, ADP-induced platelet aggregation was impaired in mice treated with BAY 11-7082. *Conclusions:* Our results suggest that NF-κB may be a novel mediator of platelet responses. The blockade of platelet function by NF-κB inhibitors might be relevant in those clinical situations where these drugs are being considered for anti-tumor and/or anti-inflammatory therapy.

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Introduction

The pleiotropic NF-κB normally exists as an inactive cytoplasmic complex whose predominant form is a heterodimer composed of p50 and p65 subunits tightly bound to inhibitory proteins of the IκB family [1]. Diverse stimuli, including cytokines, viral infection, UV radiation and free radicals, activate NF-κB through the phosphorylation of IκBs by the IKK complex. Phosphorylated IκBs are rapidly polyubiquitinated and degraded by the proteasome. Following the release from its inhibitor, NF-κB dimers translocate to the nucleus, where they bind target genes and activate transcription. Genes regulated by NF-κB include those involved in inflammation, cell survival, differentiation and proliferation responses [1]. Thus, NF-κB is an attractive target for therapeutic intervention against cancer and inflammatory diseases.

Although platelets are anucleated cell fragments, recent reports show that platelets express transcription factors including the steroid/nuclear receptors [2], peroxisome proliferator activated receptor (PPAR) β/δ [3], PPARγ [4], the glucocorticoid receptor [5] and retinoid X receptors (RXR) [6]. The interaction of the estrogen receptor β with 17β-estradiol potentiates thrombin-induced platelet aggregation [7]. PPARγ agonists prevent platelet CD40L expression and the release of CD40L, TXB_2 and ATP [4]. The binding of prednisolone to its receptor inhibits platelet aggregation [5]. RXR ligands inhibit aggregation and TXB_2 release through the inhibition of protein Gq-induced Rac activation and intracellular Ca^{2+} release [6]. Together, these findings demonstrate that transcription factors can exert non-genomic functions on platelets. While it has been previously shown that IκBα is phosphorylated and degraded after platelet activation [8], the functional significance of the NF-κB/IκB complex was not investigated. Given the importance of both NF-κB and platelets in immune cell homeostasis, inflammation and tumor progression, we have investigated the role of NF-κB on platelet physiology.

Materials and methods

Reagents

ADP, arachidonic acid (AA), epinephrine, human α-thrombin, luciferin-luciferase, ATP and TRITC-phalloidin were from Sigma (St Louis, MO, USA). Collagen was from Nycomed Pharma (Unterschleibheim, Germany). The thrombin receptor activating peptides TFLLR-NH_2 (PAR1-AP) and AYPGKF-NH_2 (PAR4-AP) were from Genbiotech (Buenos Aires, Argentina). Alexa 488-fibrinogen and Fluo-3 AM were from Invitrogen (Eugene, OR, USA). Cy3-anti-mouse IgG antibody (Ab) was from Zymed (San Francisco, CA, USA). FITC-CD62P, PAC-1 and irrelevant IgG_1 and IgM, FITC-anti-rabbit IgG, mouse anti-NF-κB p65, mouse anti-IκBα, mouse anti-actin and goat horseradish peroxidase (HRP)-anti-rabbit IgG Abs were from BD Biosciences (San José, CA, USA). Mouse anti-phospho ERK1/2 (Tyr 204) and rabbit anti-ERK were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse and rabbit anti-vWF and goat HRP-anti-mouse IgG were from Dako (Glostrup, Denmark). Rabbit anti-NF-κB p65 and rabbit anti-phospho IκBα were from Abcam (Cambridge, UK).

The NF-κB inhibitors (E)-3-[4-methylphenylsulfonyl]-2-propenenitrile (BAY 11-7082) and 6-(phenylsulfinyl)tetrazolo[1,5-*b*]pyridazine (Ro 106-9920) were from Biomol (Plymouth Meeting, PA, USA) and Tocris (Ellisville, MO, USA), respectively. Both drugs were dissolved in DMSO. The final DMSO concentration (0.5% v/v) did not have a toxic effect.

Cell preparation

Blood samples were obtained from healthy donors and drawn directly into plastic tubes containing ACD (6:1) or 3.8% sodium citrate (9:1). PRP was centrifuged in the presence of PGI_2 (75 nM) and platelets were then washed in washing buffer (140 mM NaCl, 10 mM $NaHCO_3$, 2.5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgCl_2$, 22 mM sodium citrate, 0.55 mM glucose, 0.35% BSA, pH 6.5). Finally, washed platelets (WPs) were resuspended in Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, 5 mM Glucose, pH 7.4) and typically contained ≤0.003% leukocytes. The platelet number was adjusted to 3×10^8/mL, unless otherwise stated. Where indicated, platelets were depleted from leukocytes by using a high efficiency leukoreduction filter (Purecell PL, PALL Biomedical Products, NY, USA), as previously described [4]. With this method, platelet preparations had no leukocyte contamination at all.

Mononuclear cells were isolated by Ficoll gradient centrifugation.

Immunofluorescence

WPs and leukocytes were fixed with 1% paraformaldehyde and cytospined. Cells were treated with PBS/0.1% Triton, blocked and incubated with or without (negative controls) rabbit polyclonal-anti-NF-κB or mouse monoclonal-anti-NF-κB in combination with mouse or rabbit-anti-vWF, respectively. After washing, cells were stained with FITC-anti-rabbit and Cy3-anti-mouse secondary Abs, mounted with Vectashield and visualized under fluorescent microscopy.

Immunoblotting

WPs lysates ($1–2 \times 10^9$ cells/mL) were prepared by solubilizing platelets in loading buffer (62.5 mM Tris–HCl at pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% 2-mercaptoethanol) in the presence of a protease inhibitor cocktail containing 1 mM AEBSF, 0.008 mM Aprotinin, 0.02 mM Leupeptin, 0.04 mM Bestatin, 0.015 mM Pepstatin A and 0.014 mM E-64 (Sigma). Equal amounts of proteins were electrophoresed on a 12% SDS-PAGE and electrotransferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). After blocking, the membranes were incubated overnight at 4 °C with primary Abs followed by a HRP secondary Ab. Protein bands were visualized by using the ECL reaction. Immunoblotting results were quantitated using GEL-PRO analyzer 3.1 software and values from blot reprobes were used for normalization of data for protein loads.

NF-κB ELISA assay

NF-κB DNA binding ability was measured by using an ELISA kit from Panomics (Fremont, CA, USA). In this method, activated NF-κB from cell lysates binds to a NF-κB p65 consensus binding site on a biotinylated oligonucleotide. These oligonucleotides are then immobilized on a streptavidin-coated plate. The bound NF-κB p65 is detected by a specific Ab. An additional HRP-secondary Ab provides a sensitive colorimetric readout that is quantified by spectrophotometry.

Flow cytometry

WPs were treated with the inhibitors, stimulated, fixed and incubated in PBS/0.1% FBS/CD62P or isotype FITC-IgG_1. Flow cytometry analysis was performed on a FACSCalibur flow cytometer® using CELLQUEST software (BD Biosciences). A similar technique was employed to evaluate $\alpha_{IIb}\beta_3$ integrin activation or fibrinogen binding by using FITC-PAC-1 or Alexa-488 fibrinogen, respectively.

Ca^{2+} levels were determined by loading PRP for 30 min at 37 °C with fluo-3 AM (2 μM). After centrifugation platelets were resuspended in Tyrode's buffer. To determine the amount of platelets that underwent an increase in intracellular Ca^{2+}, a threshold value was determined for each experiment in a time gate (15 s) before the addition of thrombin. Then, changes in intracellular Ca^{2+} were measured for 1 min after stimulation. Results were expressed as % of positive cells and represent the events with FL1 values above the threshold.

Platelet spreading

After incubation with NF-κB inhibitors, WPs (5×10^7/mL) were stimulated and plated on fibrinogen (150 μg/mL)-coated slides. Adhered platelets were fixed, permeabilized, stained with TRITC-Phalloidin, mounted and visualized under fluorescent microscopy.

Platelet aggregation and ATP release

Aggregation and ATP release were measured in a Lumi-aggregometer (Chrono-Log, Havertown, PA, USA). ATP levels were measured at the end of the assay by adding a known amount of standard ATP (2 μM).

Measurement of TXB_2 release

WPs were incubated for 5 min in a platelet aggregometer stirring at 1000 rpm with thrombin or AA. The reaction was stopped by the addition of ice-cold PBS/2mM EDTA/500 μM aspirin. Samples were centrifuged, and TXB_2 levels in the supernatants were measured using an ELISA kit from Cayman Chemical (Ann Arbor, MI, USA).

Platelet cytosolic phospholipase A_2 ($cPLA_2$) activity assay

WPs stimulated with thrombin were centrifuged and sonicated in ice-cold HEPES buffer (50 mM HEPES, 1 mM EDTA, pH 7.4) containing a protease inhibitor cocktail. After centrifugation (10 000 ×*g*, 15 min, 4 °C), the supernatants were stored at −80 °C until use. $cPLA_2$ activity was measured using a kit from Cayman Chemical.

Mice studies

Male BALB/c mice (18–22 g; between 8 and 10 weeks of age) were housed and bred in the animal facility at the Hematological Research Institute under the Argentine Home Office regulations for the care and use of animals. BAY 11-7082 (10 mg/kg) or vehicle was administered intraperitoneally. After 24 h the same dose was repeated and 2 h later the bleeding time was measured and blood was collected by puncture of the retro-orbital plexus.

Bleeding time

Mice were anesthetized with pentobarbital, and the tail was cut 5 mm from the distal tip and immersed in PBS at 37 °C. The bleeding time was defined as the time point at which all visible signs of bleeding from the incision had stopped.

Preparation of murine PRP

Whole blood was diluted 1:1 in PBS containing 1 mM Ca^{2+} and Mg^{2+} and centrifuged. The platelet count was adjusted to 3×10^8/mL with platelet poor plasma. Aggregation was measured as described for human platelets.

Statistical analysis

Results are expressed as means ± SEM. The Student's paired *t*-test and the Mann–Whitney *U*-test (for mice experiments) were employed to determine the significance of differences between the groups. A P value lower than 0.05 was considered to be statistically significant.

Results

Platelets contain NF-κB and platelet activation triggers IκBα phosphorylation and degradation and NF-κB release

Immunofluorescence (Fig. 1A) and Western blot studies (Fig. 1B, lane 1) show that human platelets express NF-κB. To rule out the possibility that the Western blot signal was due to leukocyte contamination, WPs were further purified by using a high-efficiency leukoreduction filter (see Materials and methods). Fig. 1(B) (lanes 4–7) shows that two different Abs directed against human NF-κB revealed a clear p65 band in leukocyte-free platelet preparations from four different donors. In addition, no signal was observed when p65 protein was analyzed using the same number of leukocytes present in WPs preparations, while a clear band was detected with higher leukocyte numbers (Fig. 1B, lanes 2 and 3).

In nucleated cells, the simplest model of NF-κB activation implies phosphorylation, ubiquitination and degradation of its inhibitor followed by binding of the released NF-κB dimers to their consensus DNA sequences [1]. Fig. 2(A–C) shows that the activation of leukocyte-free WPs with thrombin resulted in phosphorylation (Fig. 2A) and more than a 50% degradation of IκBα (Fig. 2B). Both events were prevented by pretreating the platelets with BAY 11-7082, a specific inhibitor of NF-κB activation that prevents IκBα phosphorylation [9]. Interestingly, Ro 106-9920, another NF-κB inhibitor non-structurally related to BAY 11-7082, which selectively inhibits IκBα ubiquitination [10], showed a similar inhibitory activity against IκBα degradation without modifying its phosphorylation (Fig. 2A–C).

Furthermore, ELISA assays showed that NF-κB p65 from leukocyte-free thrombin-stimulated WP lysates bound to oligonucleotides containing the DNA consensus sequence for NF-κB (Fig. 2D). This DNA binding ability was also observed in lysates from platelets stimulated with PAR1-AP and PAR4-AP, ADP, AA or collagen. Preincubation of platelets with BAY 11-7082 abrogated these responses (Fig. 2D).

Inhibition of PAC-1 binding, fibrinogen binding and platelet spreading by BAY 11-7082 and Ro 106-9920

To determine the role of NF-κB in platelet physiology, we examined functional responses in the presence of BAY 11-7082 or Ro 106-9920. As a consequence of the platelet-agonist

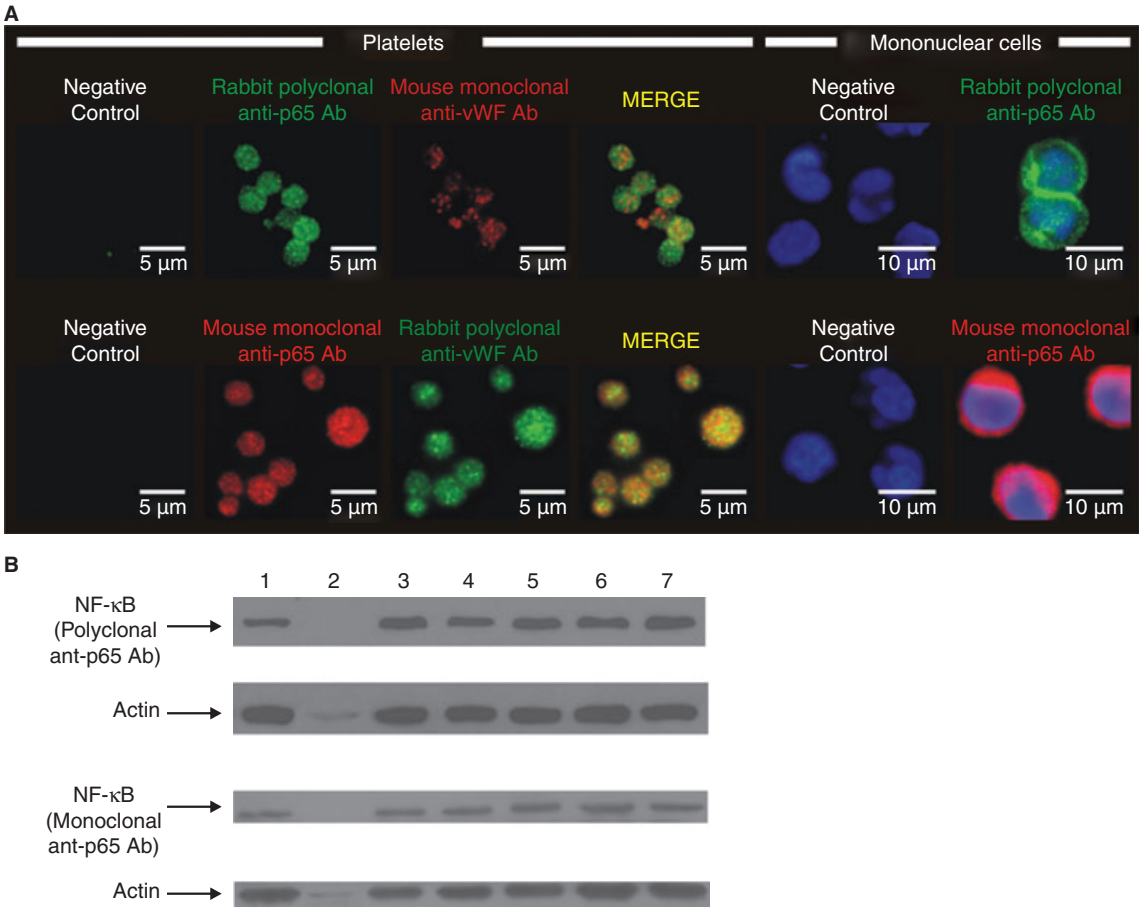

Fig. 1. Platelets express NF-κB. (A) Double immunofluorescence microscopy of resting platelets using rabbit or mouse anti-NF-κB and mouse or rabbit anti-vWF primary Abs followed by anti-rabbit-FITC/anti-mouse-Cy3 immunoglobulins. Leukocytes were stained using rabbit or mouse anti-NF-κB followed by anti-rabbit-FITC/anti-mouse-Cy3 immunoglobulins. Nuclei were counterstained with DAPI. Images are representative of three independent experiments. (B) WPs, leukocyte-free WPs (see Materials and methods) or leukocyte lysates were immunoblotted with polyclonal or monoclonal anti-NF-κB p65 Ab or with an anti-actin Ab to indicate comparable loading levels. Lane 1, WPs; lane 2, leukocytes (similar number as in the WPs sample from lane 1); lane 3, leukocytes; lanes 4–7, leukocyte-free WPs from different donor samples.

interaction, $\alpha_{IIb}\beta_3$ undergoes conformational changes allowing the exposure of its high-affinity fibrinogen-binding site (inside-out signaling); one of the resulting neoepitopes is recognized by the monoclonal Ab PAC-1. Fig. 3(A,B) shows that both thrombin-mediated PAC-1 and soluble fibrinogen binding were significantly decreased in BAY 11-7082 or Ro106-9920-treated platelets, as compared with control samples.

Fibrinogen binding results in postligand occupancy events (outside-in signaling) that lead to platelet shape change and spreading [11]. In agreement with the reduced PAC-1 and fibrinogen binding, pretreatment of platelets with the NF-κB inhibitors resulted in a marked decrease of thrombin-induced adhesion, filopodia formation and spreading of platelets (Fig. 3C).

The increase of cytosolic Ca^{2+} levels is a key early event that follows platelet stimulation [12]. Interestingly, NF-κB appears not to influence Ca^{2+} mobilization because BAY 11-7082- or Ro 106-9920-treated platelets and control platelets demonstrated an equivalent ability to increase intracellular Ca^{2+} (Fig. 3D).

Effect of NF-κB inhibitors on platelet aggregation

Figure 4 shows that WPs aggregation mediated by low thrombin concentration was markedly inhibited by BAY 11-7082 or Ro 106-9920. The decrease in PAR1-AP- or PAR4-AP-mediated aggregation by these drugs suggests that the signaling pathways triggered by both thrombin receptors are influenced by the NF-κB inhibitors.

To analyze whether the inhibition mediated by these compounds was also observed in a more physiologic environment and whether it was specific for thrombin, aliquots of PRP were stimulated with agonists that act through different mechanisms. Treatment of PRP with both NF-κB inhibitors significantly inhibited the aggregation induced by ADP, epinephrine or collagen (Fig. 5). In contrast, the aggregation response induced by AA was not modified, even when the inhibitors were added at a concentration of 100 µM. In all cases, not only was the secondary phase completely abolished, but the initial ADP- and epinephrine-induced aggregation was also significantly impaired. Increasing the concentrations of the

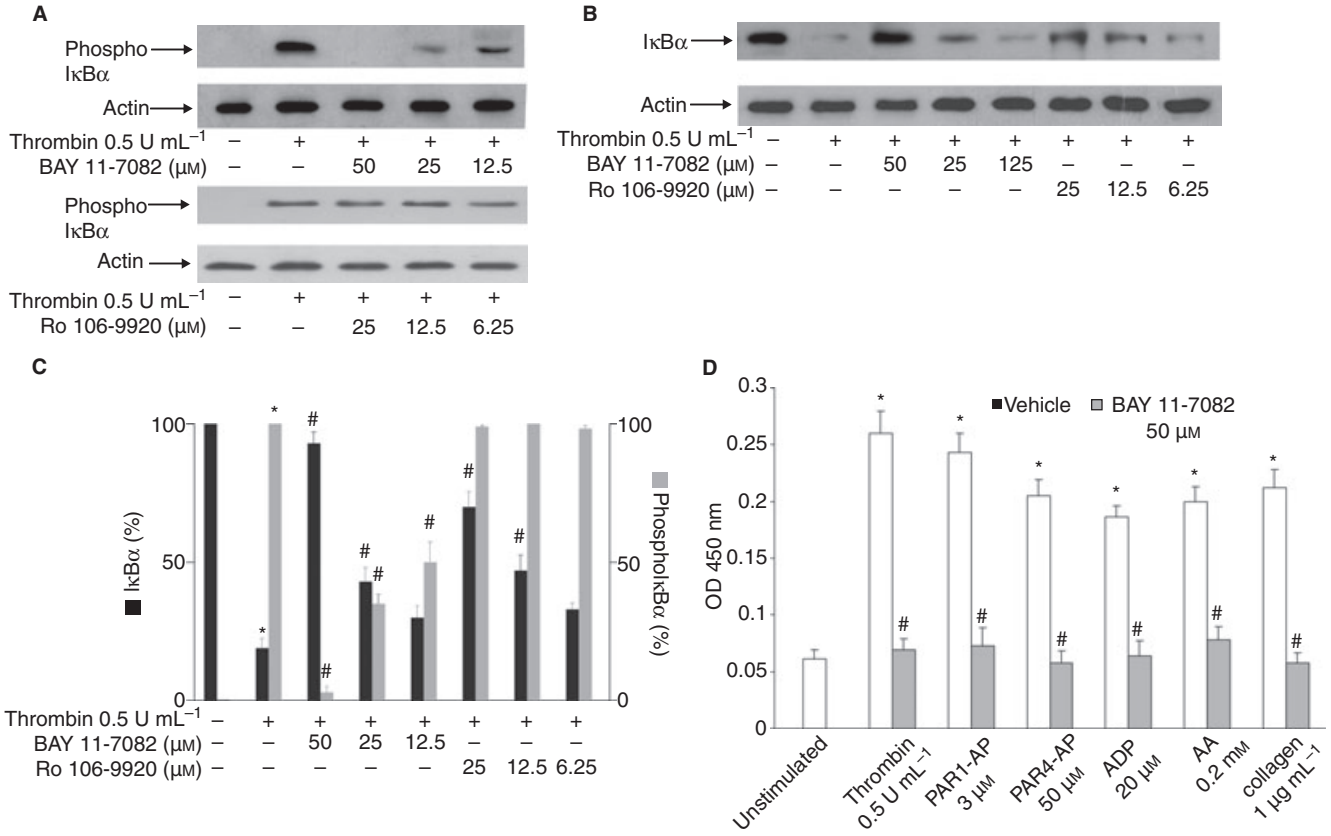

Fig. 2. Platelet agonists induce IκBα phosphorylation and degradation, and the binding of NF-κB to DNA. (A) Leukocyte-free WPs were treated with vehicle or the indicated concentrations of BAY 11-7082 or Ro 106-9920 for 1 min, and were then stimulated with thrombin for 2 min. Lysates were immunoblotted with an anti-phosphoIκBα Ab. (B) Leukocyte-free WPs were treated with vehicle or the indicated concentrations of BAY 11-7082 or Ro 106-9920 for 1 min, and were then stimulated with thrombin for 5 min. Lysates were immunoblotted with an anti-IκBα Ab. In A and B blots were reprobed with an anti-actin Ab to indicate comparable loading levels. (C) Immunoblotting results from panels A and B were quantitated using Gel-Pro Analyzer 3.1 software ($n = 4$). *$P < 0.05$ vs. unstimulated platelets; #$P < 0.05$ vs. thrombin. (D) Leukocyte-free WPs were incubated with vehicle or BAY 11-7082, stimulated with the indicated agonists and NF-κB DNA binding ability was determined using an ELISA assay. *$P < 0.05$ vs. unstimulated; #$P < 0.05$ vs. agonist alone ($n = 3$).

agonists bypassed the inhibitory action of both inhibitors (data not shown).

Effect of BAY 11-7082 and Ro 106-9920 on TXB_2 formation, ATP release and P-selectin expression

The secondary wave of aggregation induced by most agonists is mainly dependent on TXA_2 production and ADP release from dense granules. To analyze whether the inhibitory activities of BAY 11-7082 and Ro 106-9920 were related to the impairment of these responses, we estimated the levels of TXA_2 by measuring its stable metabolite, TXB_2, and measured ATP release as an indicator of dense granule secretion. Fig. 6(A) shows that the TXB_2 formation triggered by thrombin was significantly decreased in platelets that were preincubated with either BAY 11-7082 or Ro 106-9920. However, these inhibitors did not affect the conversion of exogenous AA into TXB_2. Similar results were obtained in the ATP assay (Fig. 6B). There was no ATP release in BAY 11-7082- or Ro 106-9920-treated platelets stimulated with low thrombin concentrations, while there was normal ATP release in AA-activated cells. In addition, not only dense, but also alpha granule secretion appeared to be influenced by the NF-κB inhibitors, because flow cytometry studies showed that P-selectin expression triggered by thrombin (90 ± 5% of positive cells) was reduced in BAY 11-7082- or Ro 106-9920-treated platelets (18 ± 4% and 20 ± 2% respectively; $n = 4$; $P < 0.01$ vs. thrombin).

Inhibition of platelet $cPLA_2$ activity and ERK phosphorylation by NF-κB inhibitors

The observation that NF-κB inhibitors suppressed the aggregation induced by low collagen or thrombin concentrations, as well as the secondary aggregation triggered by ADP and epinephrine, but had no effect on similar platelet responses induced by AA, suggested that the endogenous AA release could be a target for these drugs. To evaluate whether the inhibitory action was associated with an alteration in the main enzyme that accounts for the AA release, we analyzed $cPLA_2$ activity. Treatment of platelets with BAY 11-7082 or Ro 106-9920 significantly reduced the thrombin-induced enzyme activity (Fig. 7A).

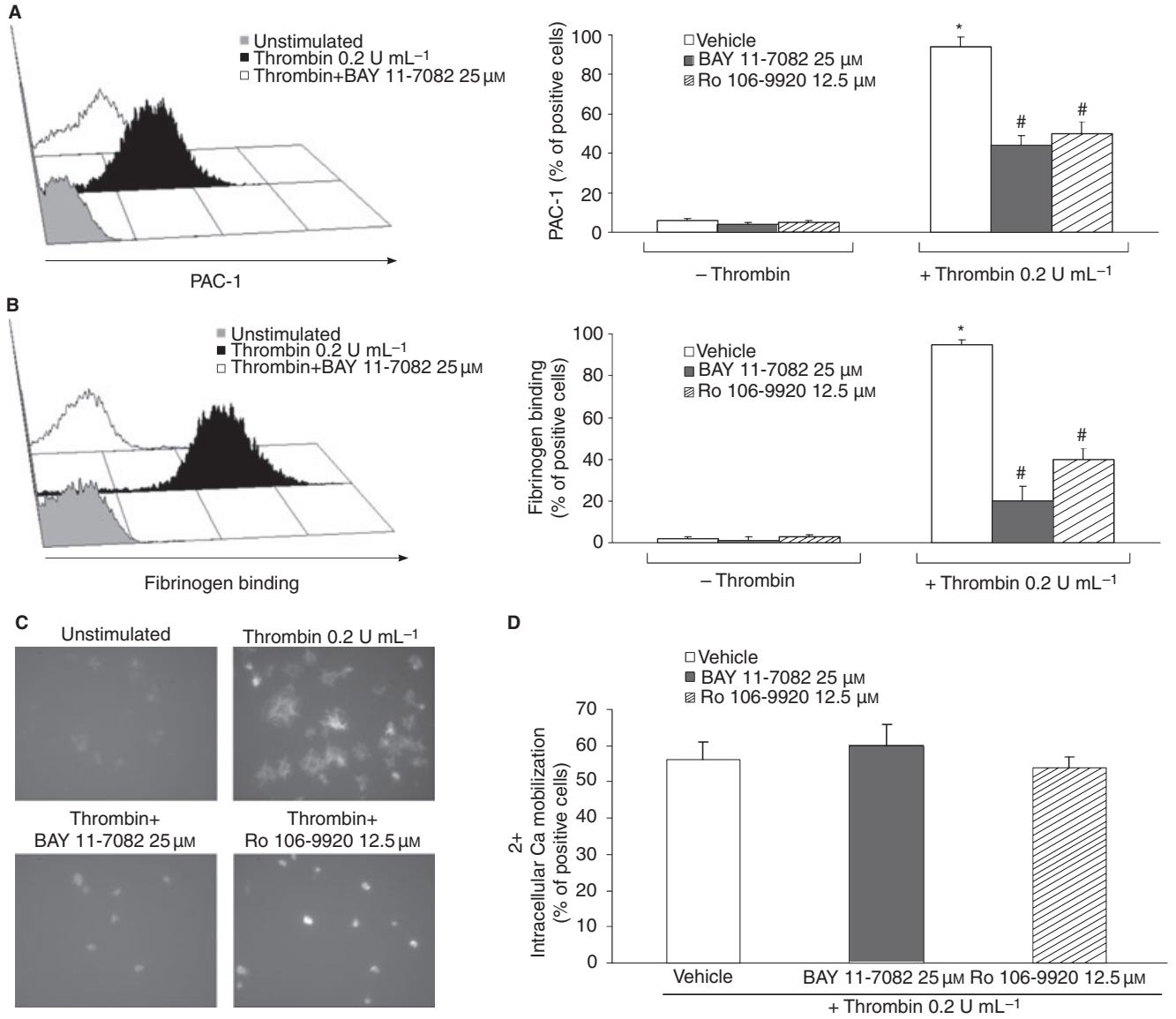

Fig. 3. BAY 11-7082 and Ro 106-9920 inhibit inside-out signaling and platelet spreading. WPs were treated with vehicle, BAY 11-7082 or Ro 106-9920 for 1 min and then stimulated with thrombin for 5 min. (A) Flow cytometry analysis of PAC-1. (B) Fibrinogen binding. Histograms are representative of five independent experiments. $^{*}P < 0.01$ vs. unstimulated platelets; $^{\#}P < 0.05$ vs. thrombin. (C) After stimulation, WPs were plated on fibrinogen-coated slides, fixed, permeabilized and stained with TRITC-Phalloidin. Platelet spreading was visualized under fluorescent microscopy (original magnification 600×). Figure is representative of four independent experiments. (D) The intracellular Ca^{2+} concentration was measured in WPs by flow cytometry using Fluo3-AM ($n = 5$).

It has been reported that upon stimulation, ERK2 is activated in human platelets [13]. In this context, phosphorylated ERK2 mediates the phosphorylation of $cPLA_2$ and increases its activity [14]. We found that thrombin-triggered ERK2 phosphorylation was markedly inhibited by BAY 11-7082 or Ro 106-9920 (Fig. 7B).

Administration of BAY 11-7082 impairs in vitro *platelet aggregation*

To determine the relevance of NF-κB inhibition *in vivo*, we evaluated the hemostatic response in mice treated with BAY 11-7082 and we found no differences in the bleeding time between treated and untreated groups. However, *in vitro* platelet aggregation was impaired in those animals that were treated with BAY 11-7082 (Fig. 8).

Discussion

Our results show that NF-κB is expressed in platelets and that platelet stimulation with different stimuli results in both the phosphorylation/degradation of the NF-κB inhibitor and the ability of NF-κB in platelet extracts to bind to DNA. Moreover, BAY 11-7082 and Ro 106-9920, two chemically unrelated NF-κB inhibitors, suppressed different platelet responses, such as cellular spreading, the inside-out signaling

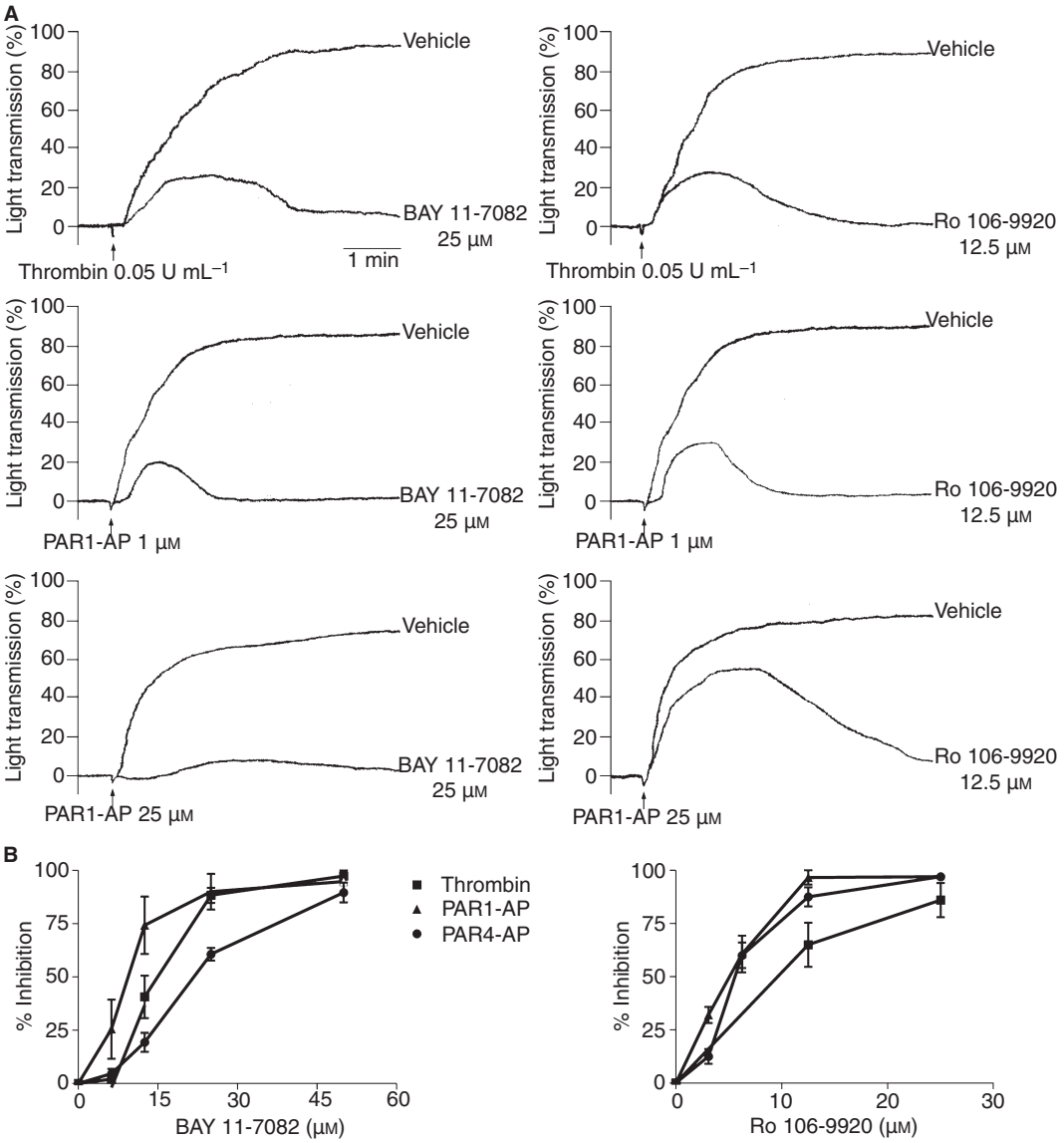

Fig. 4. NF-κB inhibitors impair platelet aggregation induced by thrombin, through inhibition of PAR1 and PAR4 signaling. (A) WPs were treated with DMSO, BAY 11-7082 or Ro 106-9920 for 1 min and then stimulated with thrombin, PAR1-AP or PAR4-AP. Results are representative of five independent experiments. (B) Concentration-response curves of BAY 11-7082 and Ro 106-9920-mediated inhibition of aggregation ($n = 4$). For each agonist and experiment, a concentration-response curve was performed and the minimal concentration of the agonist that produced an irreversible response was used to test the effect of the NF-κB inhibitors.

pathway of $\alpha_{IIb}\beta_3$ and ADP- and epinephrine-induced primary aggregation. These observations suggest that NF-κB is involved in the regulation of the initial stages of platelet activation, including the cytoskeletal rearrangements that lead to platelet shape change and the active form of the $\alpha_{IIb}\beta_3$ integrin. Although BAY 11-7082 and Ro 106-9920 suppressed the aggregation response mediated by ADP, epinephrine and low concentrations of collagen or thrombin, they failed to impair the AA-induced response. These findings indicate that the inhibitory activity of these drugs is not related to a blockade of the cyclooxigenase enzyme. In addition, the normal AA-induced TXB_2 generation indicates that the TXA_2 synthase enzyme is not a target of these NF-κB inhibitors. The failure of BAY 11-7082 and Ro 106-9920 to inhibit AA-mediated aggregation, albeit AA triggers the binding of platelet p65 subunit to DNA, indicates that this event is not required for AA-induced platelet responses. In agreement with the aggregation responses, ATP release and TXB_2 were absent after thrombin stimulation of BAY 11-7082 or Ro 106-9920-treated platelets, but were completely normal after AA stimulation. The differential effect of these drugs on platelet activation mediated by exogenous AA compared with the response elicited by weak agonists can be explained by the fact that both drugs reduced the activity of $cPLA_2$, which is the rate limiting enzyme that mediates endogenous AA release. Activation of $cPLA_2$ is regulated by both an increase in intracellular Ca^{2+} levels [15] and by the phosphorylation of p38 or ERK2 [14,16]. Surprisingly, the intracellular Ca^{2+} increase following

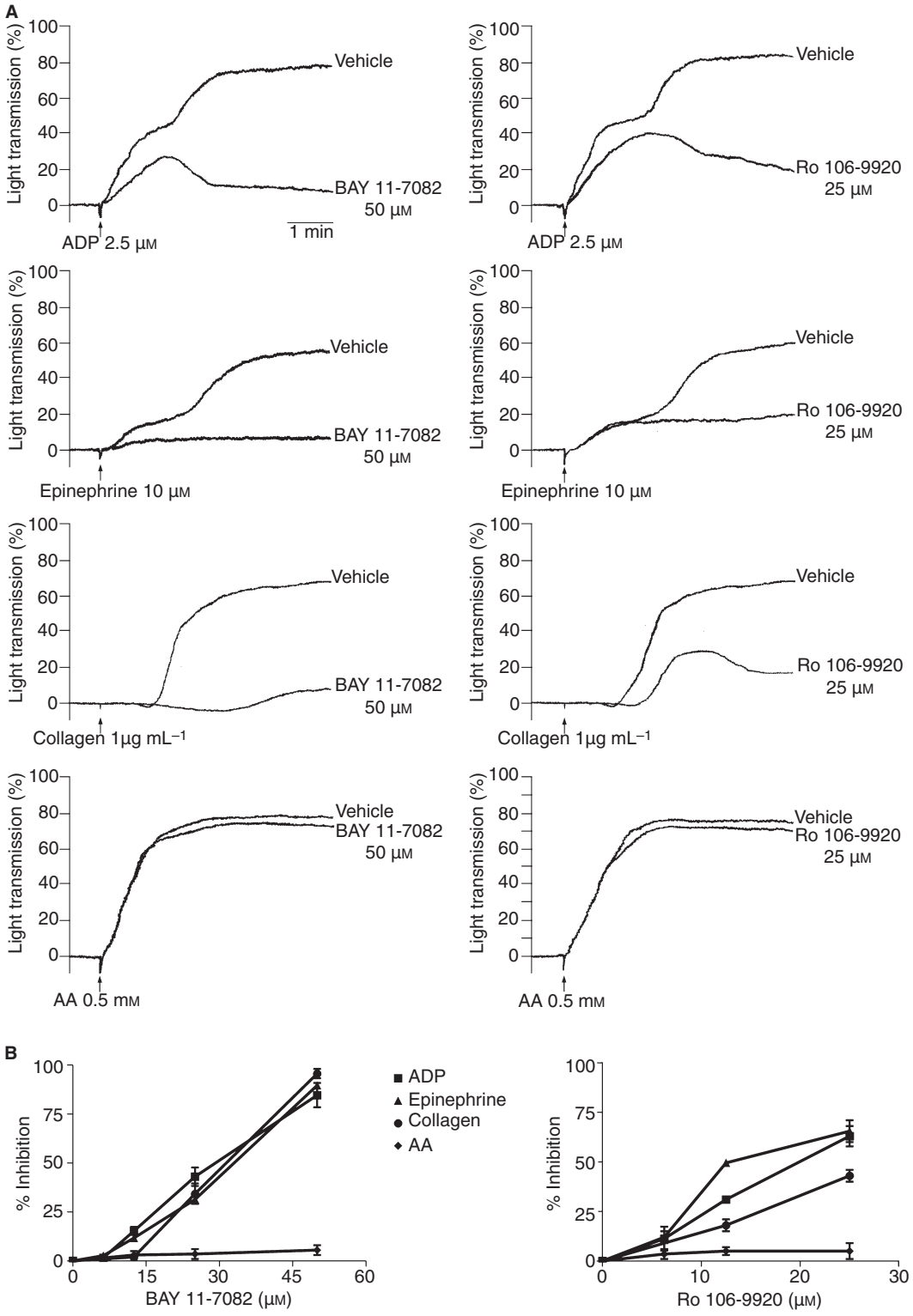

Fig. 5. Aggregation induced by ADP, epinephrine and collagen but not AA is inhibited by BAY 11-7082 and Ro 106-9920. (A) PRP was incubated with vehicle, BAY 11-7082 or Ro 106-9920 for 1 min, and then aggregation was induced with the indicated agonists. The results are representative of four independent experiments. (B) Concentration-response curves of BAY 11-7082- and Ro 106-9920-mediated inhibition of aggregation ($n = 6$).

thrombin stimulation was not modified by the NF-κB inhibitors. However, because flow cytometry does not quantify differences in Ca^{2+} concentration, and only changes in the late, sustained calcium level after stimulation can be observed, further studies are required to precisely define the effect of these drugs on Ca^{2+} levels. Interestingly, thrombin-mediated ERK

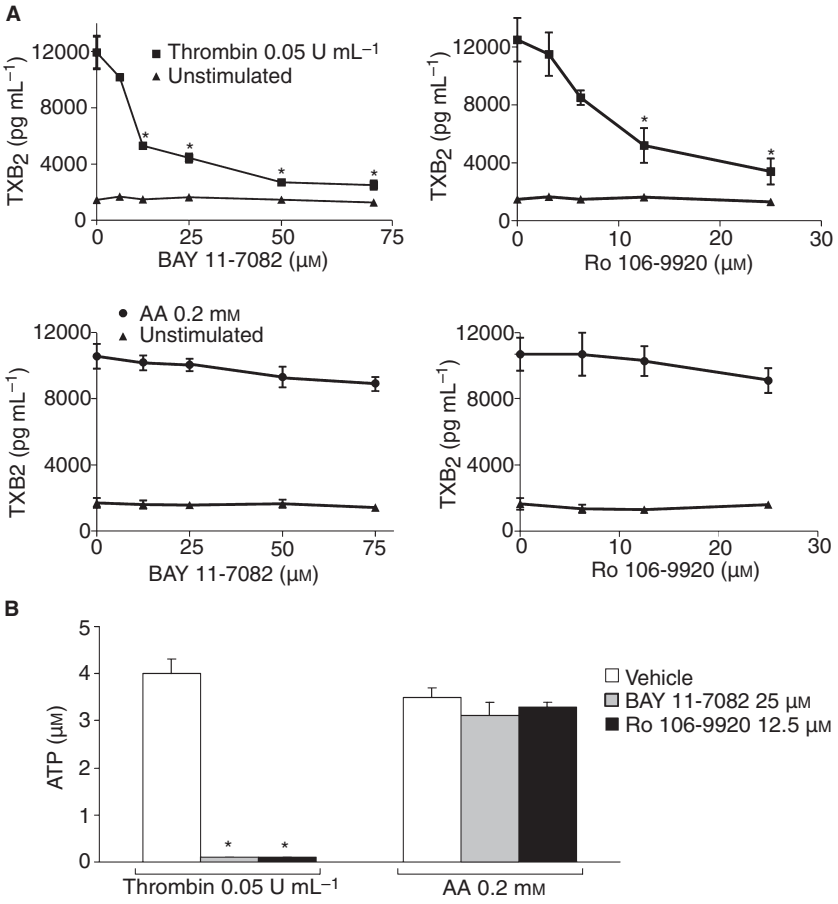

Fig. 6. BAY 11-7082 and Ro 106-9920 suppress the release of TXB_2 and ATP mediated by thrombin, but not by AA. (A) WPs were treated with BAY 11-7082 or Ro 106-9920 for 1 min, and aggregation was induced with thrombin or AA. Fibrinogen was added to the AA-stimulated samples. After 5 min, TXB_2 levels were determined by ELISA. $^*P < 0.05$ vs. thrombin alone. (B) ATP release was determined in WPs that were incubated with vehicle, BAY 11-7082 or Ro 106-9920 and then stimulated with thrombin or AA. $^*P < 0.05$ vs. thrombin ($n = 3$).

phosphorylation was markedly inhibited, suggesting that decreased $cPLA_2$ phosphorylation by ERK might account for the diminished enzyme activity. A similar pattern of altered platelet responses was recently described in a patient with an inherited $cPLA_2$ deficiency [17]. It has also been demonstrated that a pool of $cPLA_2$ is associated with $\alpha_{IIb}\beta_3$, and that the activity of $cPLA_2$ increases after fibrinogen binding [18]. Because we have also observed that NF-κB inhibitors decreased PAC-1, fibrinogen binding and spreading, a reduced $\alpha_{IIb}\beta_3$ outside-in/inside-out signaling could contribute to the reduced $cPLA_2$ activity in BAY 11-7082 and Ro 106-9920-treated platelets in addition to the reduced phosphorylation of $cPLA_2$. Because we observed that the degradation of IκBα, the release of NF-κB molecules and the inhibition of NF-κB by BAY 11-7082 and Ro 106-9920 are rapid events, any resulting effect of these phenomena most likely involves protein–protein interactions [3,4,6]. Given that $cPLA_2$ interacts with $\alpha_{IIb}\beta_3$ [18] and that NF-κB inhibitors decrease $cPLA_2$ activity, it could be speculated that the modulation of NF-κB by platelet agonists promotes the formation of a complex that includes $\alpha_{IIb}\beta_3$-NF-κB-ERK-$cPLA_2$. In this context, it has been shown that the $\alpha_{IIb}\beta_3$ receptors acquired by neutrophils through platelet-derived microparticles cooperate with β_2 integrins to activate NF-κB signaling [19]. In addition, activation of other transcription factors like estrogen receptor, sensitizes platelets to agonists by promoting the Src kinase-dependent activation of $\alpha_{IIb}\beta_3$ [7].

Platelets are widely recognized as key mediators of inflammatory responses. The expression of P-selectin on the membrane of activated platelets is the main link between platelets and inflammatory cells [20]. Interestingly, both inhibitors decreased P-selectin expression, suggesting that the non-genomic role of NF-κB in platelets could be associated with the regulation of both hemostatic- and inflammatory-mediated responses.

Finally, our data demonstrating that ADP-induced aggregation was inhibited in mice treated with BAY 11-7082 suggest that the platelet inhibition due to NF-κB suppression might also be relevant *in vivo*.

In conclusion, we have presented evidence that: (i) NF-κB is expressed in platelets; (ii) platelet agonists trigger IκBα phosphorylation/degradation and the binding of platelet NF-κB to DNA; (iii) two unrelated specific NF-κB inhibitors are capable of negatively regulating platelet spreading, aggregation and granule release through the blockade of the ERK-$cPLA_2$-

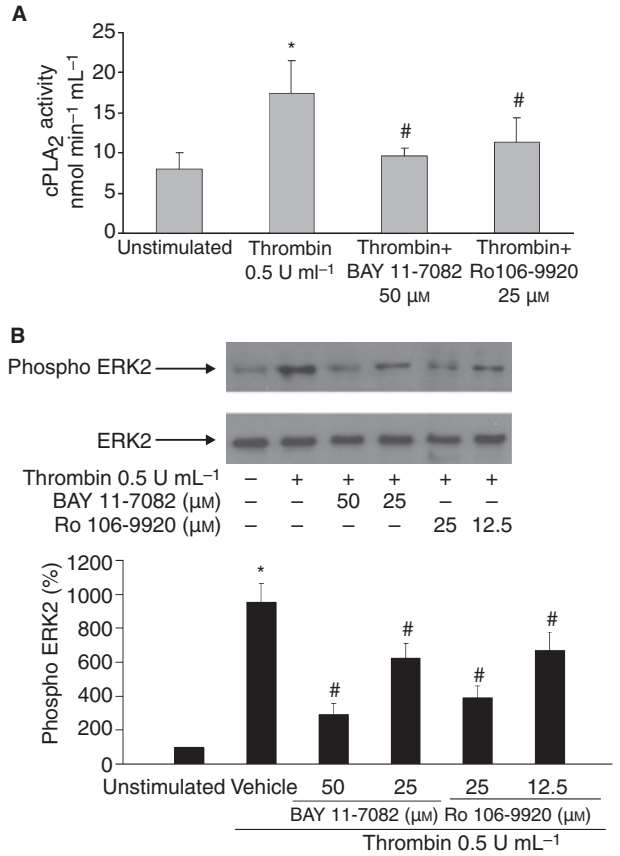

Fig. 7. NF-κB inhibitors block $cPLA_2$ activity and prevent thrombin-induced ERK2 phosphorylation. (A) WPs treated or not with the NF-κB inhibitors were stimulated with thrombin for 10 min at 37º C. Platelets were then sonicated and $cPLA_2$ activity was determined by using a $cPLA_2$ activity assay kit. $^*P < 0.01$ vs. unstimulated; $^{\#}P < 0.05$ vs. thrombin ($n = 4$). (B) WP lysates were immunoblotted with an anti-phospho ERK Ab or with an anti-ERK Ab to indicate comparable loading levels. $^*P < 0.01$ vs. unstimulated; $^{\#}P < 0.05$ vs. thrombin ($n = 5$).

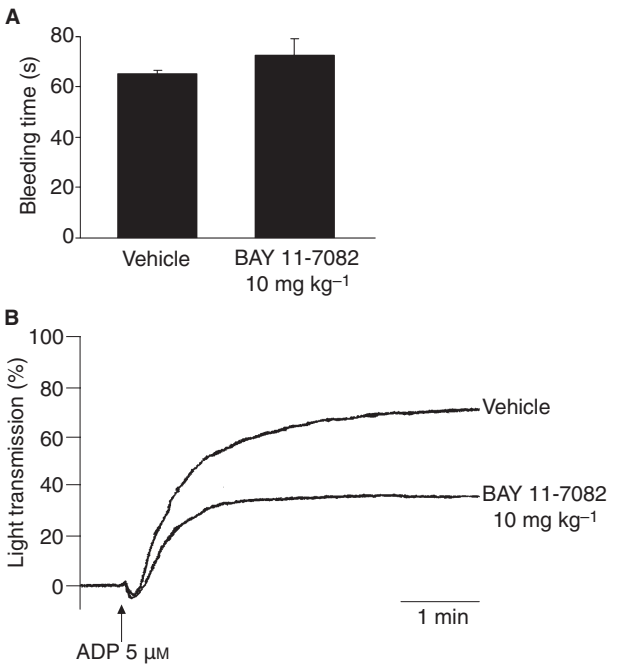

Fig. 8. Bleeding time and platelet aggregation of mice treated with BAY 11-7082. Mice were treated with vehicle or BAY 11-7082, and a second dose was administered 24 h later. Two hours after the administration of the second dose (A) the bleeding time ($n = 11$) and (B) platelet aggregation ($n = 8$) were determined. Panel B shows one representative experiment.

TXA_2 pathway; and (iv) treatment of mice with BAY 11-7082 inhibits platelet aggregation. Together, these results suggest that NF-κB could be a mediator of platelet function. Nevertheless, a usual concern about the use of pharmacological inhibitors is their potential side effects. It has been demonstrated that BAY 11-7082 does not act as a global inhibitor of cytokine-mediated phosphorylation, but selectively inhibits the phosphorylation of IκBα [9]. In addition, Ro 106-9920, despite not being structurally related to BAY 11-7082, selectively inhibits IκBα by interfering with its ubiquitination, but not its phosphorylation [10]. In fact we have evaluated platelet IκBα phosphorylation induced by thrombin in the presence of both inhibitors and as expected, BAY 11-7082 but not Ro 106-9920 inhibited IκBα phosphorylation in a concentration-dependent manner, strengthening the notion that the two drugs have different mechanisms of action. Thus, although it is highly conceivable that the inhibitory effect of these drugs on platelet function is due to a selective inhibition of the NF-κB pathway, we do not rule out the possibility that these compounds may have alternative modes of action.

The constitutive activation of NF-κB is a hallmark of many malignant tumors and accounts for profound chemoresistance. Therefore, the inhibition of NF-κB activation has been shown to be a useful strategy for increasing the sensitivity of cytostatic drug treatment. Moreover, targeting NF-κB signaling is also emerging as a potential therapy to prevent restenosis after percutaneous coronary intervention [21] and hypertension [22]. Because platelet activation is not only linked to hemostasis, but also has a relevant role in inflammation and metastasis, our present data demonstrating that NF-κB inhibitors interfere with platelet function may have a great impact when these types of drugs are considered for the treatment of cancer and various inflammatory diseases.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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